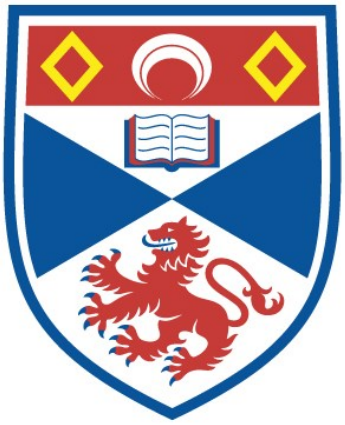
EFFECT OF THYROID STATUS ON THE CONTRACTILE AND BIOCHEMICAL PROPERTIES OF RAT SKELETAL MUSCLES

Imtiaz Majid

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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EFFECT OF THYROID STATUS ON THE CONTRACTILE AND BIOCHEMICAL PROPERTIES OF RAT SKELETAL MUSCLES

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Thesis submitted for the degree of PhD. to the

Department of Biology and Pre-clinical Medicine.

University of St. Andrews.

August, 1994.

DECLARATION

I, Imtiaz Majid, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any degree or professional qualification.

I was admitted to the faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 01/10/87 and as a candidate for the degree of PhD. on 01/10/88.

Signed I. Majid Date 16/08/94

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of PhD.

Signature of Supervisor Date
Dr. C. J. M. Nicol

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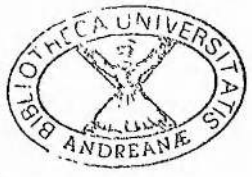

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ABSTRACT

Chemically skinned single fibres from soleus (slow) and tensor fascia latae (fast) muscles of the euthyroid rat and guinea-pig generated maximal isometric tensions in a narrow range of 125 – 150 kN/m^2 at a temperature of 25 °C and a sarcomere length of 2.75 μm.

The maximum velocity of shortening (V_{max}) of the tensor fascia latae (TFL) muscle fibres from the euthyroid rat (12.35 ± 0.95 L_oS^{-1} – Fibre lengths per second) and guinea-pig (9.93 ± 1.14 L_oS^{-1}) were 2-3 fold higher ($P < 0.05$, unpaired t-test) as compared to the soleus muscle fibres of the euthyroid rat (5.61 ± 0.54 L_oS^{-1}) and guinea-pig (3.92 ± 0.35 L_oS^{-1}) respectively. This was consistent with previous (e.g. Reiser et al., 1985a, b) results on slow and fast muscle fibres.

Storing bundles of soleus or TFL muscle fibres from euthyroid rats and guinea-pigs for short (weeks) or long term (months) resulted in significant ($P < 0.05$, unpaired t-test) reductions in both the maximum isometric tension (P_o) and V_{max} of stored single fibres in comparison to fresh single fibres. Therefore, all subsequent experiments were done using fresh single fibres.

The maximum isometric tension of chemically skinned single fibres from the soleus of mildly hyperthyroid animals (rats), was lower than that generated by equivalent fibres from mildly hypothyroid animals (127.12 ± 5.87 kN/m^2 vs. 169.02 ± 6.62 kN/m^2, $P < 0.05$, unpaired t-test). There was a corresponding reduction in tension production in TFL fibres comparing hyperthyroid with hypothyroid animals (121.30 ± 6.80 kN/m^2 vs. 142.73 ± 6.38 kN/m^2, $P < 0.05$).

On the other hand, V_{max} of single fibres from soleus muscles of hyperthyroid rats was higher than the hypothyroid counterparts (6.58 ±

0.32 L_oS^{-1} vs. 5.52 ± 0.27 L_oS^{-1}, $P < 0.05$, unpaired t-test). Similarly, V_{max} of hyperthyroid single TFL fibres was higher than the hypothyroid counterparts (13.81 ± 0.50 L_oS^{-1} vs. 11.18 ± 0.65 L_oS^{-1}, $P < 0.05$).

These differences between the hyper- and hypothyroid results – within each muscle type – were observed in the same type of fibre as identified histochemically. These were the slow oxidative (SO) fibre type from the soleus and the fast glycolytic (FG) fibre type from the TFL muscles. It is postulated that the differences in P_o and V_{max} were due to alterations in the isoforms of the myosin molecule and more specifically to the myosin light chains.

Mild dysthyreosis showed that single muscle fibres of the same histochemical type can generate heterogeneous tension/pCa (T/pCa) relationships. For example, the hyperthyroid soleus muscle fibres showed a steeper (Hill coefficient, n = 1.73 ± 0.10) T/pCa relationship which was shifted to the right with respect to the hypothyroid soleus (n = 1.54 ± 0.15) muscle fibres. The hyperthyroid TFL muscle fibres showed a steeper (n = 2.44 ± 0.28) T/pCa relationship which was shifted to the left with respect to the hypothyroid TFL (n = 1.99 ± 0.10) muscle fibres.

Acidic pH (pH 6.6) reduced maximum isometric tension relative to pH 7.0 in all four experimental groups (i.e., hyper- and hypothyroid soleus and hyper- and hypothyroid TFL muscle fibres) by 21 – 36%, $P < 0.05$, paired t-test. Similarly, V_{max} at pH 6.6 relative to pH 7.0 was also reduced in all four experimental groups by 33 – 40%, $P < 0.05$, paired t-test.

The T/pCa relationship at pH 6.6, was steeper and shifted to the right with respect to pH 7.0 in both hyper- (Hill coefficient, n = 1.73 ± 0.10 vs. n = 2.95 ± 0.43, $P < 0.005$, unpaired t-test) and hypothyroid (n = 1.54 ± 0.15 vs. n = 2.31 ± 0.38, $P < 0.05$) soleus muscle fibres.

For both the hyper- and hypothyroid TFL muscle fibres, the T/pCa relationship was shifted to the left at pH 6.6 with respect to pH 7.0. The hyperthyroid TFL muscle fibres generated a shallower T/pCa relationship at pH 6.6 with respect to pH 7.0 ($n = 2.01 \pm 0.39$ vs. $n = 2.44 \pm 0.28$ respectively). Whereas, the hypothyroid TFL muscle fibres had a steeper T/pCa relationship at pH 6.6 with respect to pH 7.0 ($n = 2.26 \pm 0.29$ vs. $n = 1.99 \pm 0.10$ respectively), although the values were not significantly different.

At increased inorganic phosphate (P_i) concentration (7.5mM) the maximum isometric tension was slightly reduced, relative to a P_i concentration of 0.0mM, in all four experimental groups (i.e., hyper- and hypothyroid soleus and hyper- and hypothyroid TFL muscle fibres) by 1 - 12%, $P < 0.05$ paired t-test, for the two hypothyroid groups only. On the other hand, V_{max} was relatively unchanged at 7.5mM P_i with respect to 0.0mM P_i.

At 7.5mM P_i, the T/pCa relationship was steeper and shifted to the right with respect to 0.0mM P_i, in both hyper- ($n = 1.73 \pm 0.10$ vs. $n = 2.87 \pm 0.13$, $P < 0.005$, unpaired t-test) and hypothyroid ($n = 1.54 \pm 0.15$ vs. $n = 2.54 \pm 0.41$, $P < 0.05$) soleus muscle fibres.

Similarly, at 7.5mM P_i, the T/pCa relationship was steeper and shifted to the right with respect to 0.0mM P_i, in both hyper- ($n = 2.44 \pm 0.28$ vs. $n = 2.99 \pm 0.19$) and hypothyroid ($n = 1.99 \pm 0.10$ vs. $n = 3.22 \pm 0.13$, $P < 0.005$, unpaired t-test) TFL muscle fibres.

The rightward shift in the T/pCa relationship was larger in the soleus than TFL muscle fibres, suggesting the former are more sensitive to increased inorganic phosphate concentration than the latter.

On the whole muscle level (within each muscle type), the Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity was higher in hyper- than hypothyroid soleus muscles (0.176 ± 0.016 vs. 0.097 ± 0.009 μmoles of P_i/mg of protein/min, $P < 0.05$, unpaired t-test). Similarly, for the TFL muscles, the

Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity was higher in hyper- than hypothyroid animals (0.418 ± 0.037 vs. 0.359 ± 0.039 μmoles of P_i/mg of protein/min).

In soleus muscles, this corresponded to a higher percentage (32.96 ± 4.54) of fast oxidative glycolytic (FOG) fibres in the hyperthyroid state and conversely a higher percentage (89.03 ± 2.60) of slow oxidative (SO) fibres in the hypothyroid state. In the TFL muscles, this corresponded to higher percentage (85.89 ± 1.62) of fast glycolytic (FG) fibres in the hyperthyroid state and conversely a higher percentage (20.23 ± 2.31) of FOG fibres in the hypothyroid state.

Collectively these data suggest that mild hypo- and hyperthyroidism changes the dynamic equilibrium of the myofibrillar (regulatory and contractile) proteins from slow to fast in the direction hypo- to hyperthyroid and from fast to slow in the direction hyper- to hypothyroid, irrespective of whether a slow or fast muscle is used.

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INTRODUCTION

OVERVIEW OF SKELETAL MUSCLE CONTRACTION AND RELAXATION

Nerve impulses reaching the neuromuscular junction cause the release of acetylcholine from the nerve endings, by causing a change in the voltage across the membrane of the nerve cell. This neurotransmitter, in turn, initiates a change in the membrane voltage of the muscle. This event is followed by the process of excitation–contraction (E–C) coupling.

E–C coupling is the process by which excitation of the muscle cell membrane rapidly spreads into the fibre interior along membrane invaginations that form the tansversal tubular system and induces calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR) [for reviews see Ebashi, 1976; Rios et al., 1992). The released Ca^{2+} acts on the regulatory proteins controlling cross bridge cycling and ultimately contraction.

Contraction of skeletal muscle is regulated by the regulatory proteins located in the thin filaments, viz., troponin (Tn) and tropomyosin (Tm). Tn consists of three different subunits (Greaser & Gergely, 1973) termed troponin T (Tn T), troponin I (Tn I) and troponin C (Tn C).

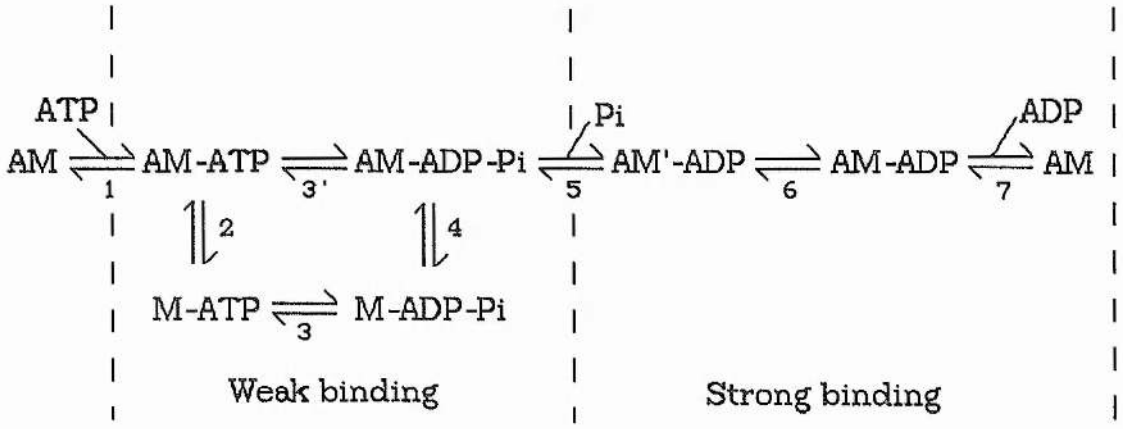
Tn T is the Tm binding subunit, Tn I is an inhibitory subunit of the tension generating interaction between actin and myosin that uses the energy liberated by adenosine triphosphate (ATP) hydrolysis. And Tn C is a calcium binding subunit.

The popular view is that upon binding Ca^{2+} to Tn C the inhibitory action of Tn I is removed due to a lateral shift of the Tn-Tm complex, which eliminates the inhibition of the cross bridge cycling between actin and myosin (steric blocking model) (for reviews see Perry, 1979; Ebashi, 1980).

However, it has been proposed that Ca^{2+} binding to troponin does not itself move Tm but instead allows it to be displaced more easily by strongly bound cross bridges (for review see El-Saleh et al., 1986).

The current view of the cross bridge cycle is that during its cyclic interaction with actin (A), the myosin (M) cross bridge binds either weakly or strongly to actin depending on the nucleotide bound to the active site (Fig. 1.1).

Fig. 1.1: Schematic model (presented from Metzger & Moss, 1990b) of the kinetics of the actomyosin ATP hydrolysis reaction during contraction in skeletal muscle, where A is actin and M is heavy meromyosin or myosin S1 and AM is actomyosin. The state AM'-ADP, which is capable of binding P_i, is formed preferentially during ATP hydrolysis. AM'-ADP is different from AM-ADP (formed by adding ADP to AM) in that AM-ADP cannot readily bind P_i.



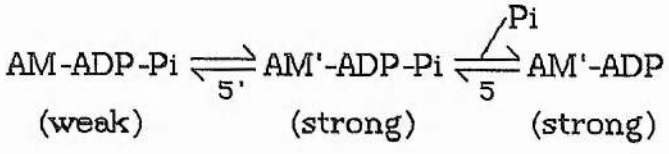
If ATP or the products of hydrolysis are bound (M-ATP, M-ADP-P_i), myosin rapidly attaches to and detaches from actin and is thus weakly bound. Upon release of inorganic phosphate (P_i), it is believed that the working stroke occurs as the cross bridge undergoes a conformational change to a state that is more strongly bound to actin. This transition results in tension and/or actin movement. Following adenosine diphosphate (ADP) release, myosin remains strongly bound to actin on a rigor bond (AM) until another molecule of ATP binds and detaches the

head, and the cycle begins again. This reaction scheme is adapted from current models of the ATP hydrolysis pathway in skeletal muscle (Taylor, 1979; Eisenberg & Hill, 1985; Hibberd & Trentham, 1986; Goldman & Brenner, 1987).

The strongly bound high tension state (AM'-ADP) is likely to be the dominant cross bridge form during maximum isometric tension (Metzger & Moss, 1990b). Whereas, the maximal velocity of shortening (V_{max}) in skeletal muscle is obtained during unloaded contractions where the number of strongly bound, high tension states of the cross bridge is low and the overall cycle rate is maximal. In contrast to the maximal rate of tension development, where the rate of cross bridge attachment appears to be limiting (Brenner, 1987; Metzger et al., 1989), V_{max} is thought to be limited by the rate of cross bridge dissociation. The rate limiting step in cross bridge detachment is unknown, but the possibilities include steps 6,7,1 and 2 in the scheme shown in Fig. 1.1.

Recently, an additional step to the cross bridge cycle has been proposed, where the weak AM-ADP-P_i cross bridge state isomerises to form a strong AM'-ADP-P_i cross bridge state (Fig. 1.2) which can produce either a similar tension to the AM'-ADP state (Millar & Homsher, 1990) or produces less tension than the AM'-ADP state (Martyn & Gordon, 1992)

Fig. 1.2: The following additional step has been proposed recently for the scheme presented in Fig. 1.1. In this model P_i release takes place in two steps. The weak AM-ADP-P_i cross bridge first isomerises to form a strong AM'-ADP-P_i cross bridge that can produce tension. P_i is then released to form a strong AM'-ADP cross bridge.



Relaxation occurs when the stimulus initiating the contraction is removed. Therefore, no further Ca^{2+} is released from the SR. The Ca^{2+} which is present in the cytosol is actively taken back into the SR by the Ca^{2+} ATPase pump of the SR. Thus the intracellular Ca^{2+} concentration falls significantly to relieve the troponin molecule of its bound Ca^{2+} (for reviews see Inesi, 1985; Ruegg, 1988). In fact, simultaneous Ca^{2+} ATPase re-uptake by the SR competes with Tn binding of calcium (Gillis et al., 1982).

In addition to the SR, there are other Ca^{2+} binding proteins present in the cytosol, such as parvalbumin, calmodulin and the (phosphorylatable) myosin light chain, which facilitate relaxation (for reviews see Gillis, 1985; Ruegg, 1988). Parvalbumin is normally present in higher concentrations in fast twitch skeletal muscles than slow twitch muscles (Heizmann, 1984; Gillis, 1985).

MYOSIN HEAVY AND LIGHT CHAINS

The contractile protein myosin is a hexameric molecule consisting of two heavy chains and two pairs of light chains which are associated with the globular head of the heavy chain (Lowey & Risby, 1971; Weeds, 1976). Myosin exists as multiple isoforms as a result of polymorphic expression of both the myosin heavy chain (MHC) and myosin light chain (MLC) subunits. These myosin isoforms differ structurally in either their heavy chain primary structure or by the type of light chain component associated with the heavy chain or by both (e.g., slow vs. fast myosin) (Staron & Pette, 1987a).

In the rat, MHC has been shown to exist as a large family of protein isoforms that are encoded for by a highly conserved multigene family (for review see Sywnghedauw, 1986). These are the cardiac α- and ß-MHC genes; the skeletal embryonic, neonatal, fast type IIA, IIB and IID, and the slow tonic MHC genes; and an extra-ocular and a superfast muscle specific MHC genes. (Izumo et al., 1986; Pette & Staron, 1993). In addition, the cardiac ß-MHC and the skeletal muscle type I MHC protein isoforms have been shown to be encoded by the same cardiac ß-MHC gene (Lompre et al., 1984).

Of the ten MHC genes known to exist, three are predominantly expressed in adult rat skeletal muscle and are designated as fast type IIB, fast type IIA and slow type I MHC. These genes have been related to the three myosin heavy chain isoforms (Table 1.1), viz. type IIB (FG), IIA (FOG), and I (SO) respectively (Tsika et al. 1987; Diffee et al., 1991).

Table 1.1: Myosin heavy chain isoforms in adult extrafusal fibres.

	SLOW	FAST
Myosin heavy chain	type I (= cardiac ß-MHC) cardiac α–MHC, and slow tonic MHC	type IIA, IID, IIB, extra-ocular, superfast, embryonic and neonatal MHC

The MHC isoforms have been assigned to their respective native isomyosins, SM_1 and SM_2 (type I MHC i.e. SO type); IM (type IIA MHC i.e. FOG type) and FM_1, FM_2, FM_3 (type IIB i.e., FG type) (Tsika et al., 1987, Fitzsimons et al., 1990). Moreover, IM consists of a mixture slow and fast light chains, whereas SM and FM consist solely of slow and fast light chains respectively (Tsika et al., 1987).

In adult skeletal muscles, there are two classes of light chains. These have become known as the alkali light chains and the phosphorylatable (P- or DTNB) light chains. They are so termed because the former can be removed from the myosin molecule under alkaline conditions whereas, the latter can be removed from myosin by reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and can be phosphorylated (Manning & Stull, 1982). It has been shown that removal of alkali light chains leads to loss of adenosine triphosphatase (ATPase) activity, whereas the P-light chains can be removed without the loss of ATPase activity (Perrie et al., 1973). The myosin light chains are identified by a number which corresponds to their migration on sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis. A low number corresponds to a slower migration relative to a higher number which corresponds to a faster migration.

In slow muscles, there are two alkali light chains (Table 1.2) termed MLC_{1sa} and MLC_{1sb} and there is one P-MLC termed MLC_{2s}. Similarly, there are two alkali light chains in fast muscle, termed MLC_{1f} and MLC_{3f}, and one P-MLC, termed MLC_{2f} (for review see Barton & Buckingham, 1985). Generally the slow MLC isoforms predominate in slow muscles and the fast MLC isoforms predominate in the fast muscles (Lowey & Risby, 1971; Weeds, 1976).

Table 1.2: Myosin light chain isoforms in adult extrafusal fibres.

Myosin light chains	SLOW	FAST
Alkali	MLC_{1sa}, MLC_{1sb}	MLC_{1f}, MLC_{3f}
Phosphorylatable	MLC_{2s}	MLC_{2f}

DIFFERENTIATION AND PLASTICITY OF SKELETAL MUSCLE

Mammalian skeletal muscle fibres undergo postnatal differentiation into slow and fast types (for reviews see Buchtal & Schmalbruch, 1980; Jolesz & Sreter, 1981; Swynghedauw, 1986). At birth, there is little difference in the contraction speed of muscles that will become fast or slow later in life (Close, 1964). In the rat, all fibres are uniformly and multiply innervated early in development and this polyneural innervation regresses to a monosynaptic innervation by the end of the second week after birth (Butler-Browne & Whalen, 1984).

During rat skeletal muscle development, several isomyosin transitions take place. In muscle fibres, destined to contain adult fast-type myosin, sequential appearance of embryonic and neonatal myosin isoenzymes is found before the adult form becomes the predominant isoenzyme (Whalen et al., 1981; Butler-Browne et al., 1982). In muscles, such as the soleus, a study (Butler-Browne & Whalen, 1984) has shown that two major types of isomyosin are present in the developing soleus muscle in the early postnatal period. One type contains adult slow myosin by one week after birth, whereas the other type undergoes isoenzyme transitions similar to developing fast fibres. These fast fibres are gradually converted to slow fibres over a period of several months. Studies have mainly concentrated on the isomyosins and it is not known whether there are embryonic forms of regulatory proteins (Swynghedauw, 1986).

When the myosin molecule is analysed for its MHC content the sequential appearance of the different isoforms of myosin heavy chains in both slow and fast skeletal muscle, is similar to the pattern observed for the isoenzymes of myosin (Swynghedauw, 1986). In addition a specific embryonic nonphosphorylatable MLC has been found in rat fast skeletal

muscle (Whalen et al., 1978).

The sequential appearance of myosin isoenzymes supports the concept that developing muscle fibres will undergo a pre-programmed sequence of myosin transitions. However, there is evidence that motorneurones and hormones, especially thyroid hormones, assume an important function in regulating myosin protein isoforms during development; and that, in their absence, the normal expression of diversity is perturbed (Butler-Browne et al. 1984; Rubinstein et al. 1988).

The importance of innervation in differentiation of fibres is clearly illustrated by 'cross-innervation' experiments in which fast muscles can be transformed into slow ones and vice versa and it is principally the frequency of motorneuron discharge which govern these changes (for review see Buchtal & Schmalbruch, 1980). Continued innervation seems to be required for the maintenance or the induction of slow myosin during development (Jolesz & Sreter, 1981) but the appearance of adult fast myosin does not require innervation (Butler-Browne et al. 1982).

Thyroid hormone levels also exert a major influence on myosin isoenzyme transitions in developing animals (Gambke et al., 1983; Butler-Browne et al., 1984; Rubinstein et al. 1988). Hypothyroidism slows the endogenous program in both slow and fast muscles. In fast muscles, neonatal myosin remains predominant in hypothyroid animals during the first month of their life, and only traces of adult fast myosin can be detected. The soleus muscle of hypothyroid animals contains adult slow isoforms, but it also has an abnormally high amount of neonatal myosin. On the other hand, hyperthyroidism allows muscle development to occur precociously. It is not yet possible to decide whether this is a direct result of the effects of the hormone on the development of fibres or an indirect consequence of retardation in the maturation of the central nervous system (Butler-Browne et al. 1984).

Alternatively, thyroid hormone may exert its influence by effects both on the muscle fibre and on the developing motorneuron (Rubinstein et al., 1988).

Adult skeletal muscle, while preserving its specific tissue characteristics, can respond in a plastic manner to a variety of stimuli. Several factors have been identified that influence the differentiated character of skeletal muscle fibres. These include innervation (e.g., slow to fast or fast to slow conversion), physiological demand and thyroid hormones. Whatever, procedure brings about the transformation, it results in changes in contractile protein isoforms, alterations in the levels of glycolytic versus oxidative metabolic enzymes, switches in proteins of the sarcoplasmic reticulum, and modulation of a wide variety of additional properties (Pette, 1980; Buchtal & Schmalbruch, 1980; Swynghedauw, 1986).

CONTRACTILE PROPERTIES OF SLOW AND FAST SKELETAL MUSCLES

Slow and fast mammalian skeletal muscles can be distinguished in physiological experiments by differences in the properties of their isometric and isotonic contractions (Close, 1972). Contraction characteristics of slow and fast mammalian muscles have been examined in a number of studies (Close, 1964; Close, 1969; Gulati, 1976; Ranatunga, 1977, 1982). These studies have shown on the basis of measurements such as twitch contraction time, rate of rise of tetanic tension and shortening velocity, that the contraction speed of fast muscle is 2–3 times higher than in slow muscle. This reflects the higher myosin ATPase activity of fast muscles, since velocity of shortening has been found to correlate with the actin and Ca^{2+} activated myosin ATPase activity in different muscles of a variety of animal species (Barany, 1967).

Slow and fast muscles may be further distinguished by metabolic and histochemical characteristics into three major fibre types from which they are composed (Peter et al., 1972; Ariano et al., 1973; Armstrong & Phelps, 1984). The fibres types are slow oxidative (SO, type I), fast oxidative glycolytic (FOG, type IIA) and fast glycolytic (FG, type IIB).

The SO fibres contain type I MHC and fast fibre types FOG and FG contain type IIA MHC and type IIB MHC respectively (Danieli-Betto et al., 1986; Staron & Pette, 1987a, b).

In addition to these three major fibre types, Staron and Pette, 1987a have identified two other fibre types in slow muscles, viz., type IC and IIC. These seem to be transitional fibres, with the IC type fibres being histochemically similar to SO fibres and the IIC type fibres being histochemically similar to FOG fibres. In fast muscles, Staron and Pette, 1987b have also identified a transitional fibre type (type IIAB) which is histochemically between FOG and FG fibres.

Generally, SO fibres exhibit a low myosin ATPase activity, a moderate oxidative capacity and a low glycogenolytic capacity. Whereas FOG fibres a high myosin ATPase activity, a high oxidative capacity and a moderate to high glycogenolytic capacity. And FG fibres a moderate to high myosin ATPase activity, low oxidative capacity and a high glycogenolytic capacity.

The properties of each fibre indicate its probable function. For example, FG fibres would function as fast units for short-term powerful phasic activity because they fatigue quickly, whereas FOG fibres would be better adapted for sustained phasic activity, these fatigue at a slower rate than FG fibres. On the other hand, the SO fibres are low speed economical contractile units suitable for sustained tonic activity, because they show little fatigue (Close, 1972). Fibres within skeletal muscles are organised into motor units, each fibre within a motor unit has the same histochemical and metabolic characteristics (Jolesz & Sreter, 1981).

On the single fibre level, the maximum shortening velocity (V_{max}) of a single muscle fibre is a characteristic property (Julian et al., 1986) and is also correlated with the rate of ATP hydrolysis by myosin contained within it (Edman et al., 1988). Experiments on skinned muscle fibres have shown similar differences in V_{max} between slow and fast muscle fibres as in whole muscles and a difference also exists between the two types of fast fibres, with FG fibres having a higher V_{max} value than FOG fibres (Reiser et al., 1985 a, b; Sweeney et al., 1986, 1988).

However, with respect to the maximal isometric tension generating capacity of slow and fast mammalian muscles, a confused picture exists. First of all, a range of values have been reported 100 – 490 kN/m^2 (Close, 1972; Powell et al., 1984). Secondly, on both the whole muscle and the

single fibre level, it has either been shown that no significant difference exists in the tension generated by slow and fast muscles/fibres (Takagi & Endo, 1977; Nicol & Bruce, 1981; Metzger & Moss, 1987; Reiser et al., 1987a, b; Greaser et al., 1988; Sweeney et al., 1988; Ranatunga & Thomas, 1990) or fast muscles/fibres generate higher tensions than their slower counterparts (Close, 1972; Stephenson & Williams, 1981; Powell et al., 1984; Mounier et al, 1989). Additionally a single fibre study (Gardetto et al., 1989) has reported tension values higher in slow than fast fibres. As far as the tension between the two fast fibre types is concerned this has been shown to be similar between FOG and FG fibres where these have been identified (Sweeney et al., 1988; Gardetto et al., 1989).

EFFECT OF TEMPERATURE AND SARCOMERE LENGTH

Twitch tension in mammalian muscle tends to be largely independent of temperature or decreases with increasing temperature according to the predominant muscle fibre type in the muscle under investigation. It generally remains constant in slow twitch (e.g. soleus) muscles but decreases with increasing temperature in fast twitch (e.g. EDL – extensor digitorum longus) muscles (Close & Hoh, 1968; Close, 1972; Hoh, 1974; Issacson et al., 1970; Ranatunga, 1977, 1980; Buller et al., 1984). The basis of this differential thermal sensitivity between fibre types is not known. Moreover, its functional significance is unclear, particularly because these muscles, soleus and EDL, normally function tetanically in vivo. Results for single intact muscle fibres (Lannergren & Westerbald, 1987) and for maximal tension in Ca^{2+} activated single skinned fibres (Stephenson & Williams, 1981) show a thermal dependence similar to that reported for whole muscle preparations.

Maximal tetanic tension, on the other hand, has a low positive

thermal dependence which is independent of the fibre type (Close, 1972; Issacson et al., 1970; Ranatunga, 1977, 1980).

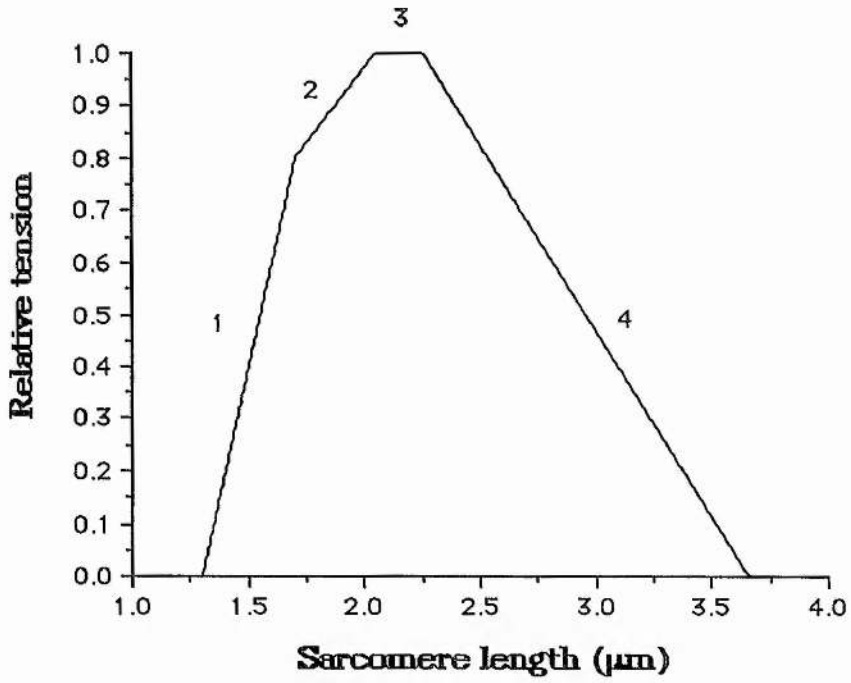
V_{max} has been reported either to have a greater thermal dependence (i.e., V_{max} increases with increasing temperature) in fast than in slow twitch muscle (Faulkner, 1980) or both types of muscles have similar thermal dependence (Ranatunga, 1982, 1984).

One feature of the cross bridge theory proposed by Huxely (1957) is that cross bridges function as independent tension generators. This leads to the prediction that the tension generating capacity of skeletal muscles is directly proportional to the overlap of thick (myosin) and thin (actin) filaments. The relationship between isometric tension and sarcomere length (SL) in intact single muscle fibres from the frog has been well characterised in tetanically stimulated skeletal muscle fibres in which SL was servo-controlled (Gordon et al., 1966a, b).

Tension (Fig. 1.3) is found to be independent of length over a narrow range of lengths near the optimum because the number of tension generating sites does not change (the plateau region), this corresponds to the bare zone in the middle of the myosin filament. At sarcomere lengths above the optimum for tension development tension decreases (the descending limb of the length tension relation) due to a reduction in amount of overlap of thick and thin filaments and a consequent decrease in the number of myosin cross bridges interacting with actin. At sarcomere lengths below optimum, tension falls (the shallow part of the ascending limb) due to overlap of thin filaments with cross bridges on the opposite end of the thick filament and at still shorter lengths (the steep part of the ascending limb) due to a resting tension resulting from the compression of thick filaments against the Z-lines.

Fig. 1.3: Graphical representation of the isometric tension–sarcomere length relation of single fibres from frog semitendinosus muscle, obtained by Gordon et al., 1966a, b.

1. Steep ascending limb
2. Shallow ascending limb
3. Plateau region
4. Descending limb



With only a few variations, which can be explained on the basis of SL non-uniformities along the fibres, similar length tension relationships have been obtained in maximally Ca^{2+} activated skinned preparations of amphibian muscles (Schoenberg & Podolsky, 1972; Moss, 1979; Julian & Moss, 1980), mammalian skeletal (Stephenson & Williams, 1982; Allen & Moss 1987) and cardiac muscles (Fabiato & Fabiato, 1978; Kentish et al., 1986).

However there is still inadequate information concerning the length dependence of the fibres ability to produce motion. Studies on frog single muscle fibres (Huxley & Julian 1965; Gordon et al., 1966a, b) found the speed of shortening against a small load (4-6% of tetanic tension) fell by approximately 15% during shortening from 3.0 to 2.0μm SL, though the initial speed of shortening, measured at 3.0 and 2.5μm SL, did not differ appreciably. A detailed study by Edman (1979) using

the slack test method to measure V_{max} of frog single muscle fibres found that in the SL range 1.65 – 2.7μm, V_{max} was constant. At SL < 1.65μm V_{max} showed a decrease, in this region of sarcomere lengths the effect on V_{max} is believed to be a result of tension within the fibre resisting shortening. And at SL > 2.7μm V_{max} showed an increase, which was found to be related to the resting tension of the fibre. Unstimulated fibres develop passive tension over this range of lengths and can shorten rapidly. The passive tension seems to act as compressive tension (i.e. directed towards the centre of the fibre) enhancing V_{max} above the value attained at zero load. It should be noted, in vertebrate muscle the length of the thick filament is the same in all species that have been investigated (Page & Huxely, 1963). The best estimate of this length is 1.56 – 1.60μm (Close, 1972; Craig & Offer, 1976). The length of the thin filament, however is different in different species, varying from 1.98μm (frog) to 2.38μm (rat), (Page & Huxely, 1963; Close, 1972; ter Keurs et al., 1981).

TENSION-pCa (T/pCa) RELATIONSHIP

It is known that interesting and important information about the Ca^{2+} regulatory proteins of skeletal muscle can be obtained by activating single skinned muscle fibres with different Ca^{2+} concentrations, since the regulatory proteins are believed to be involved in the Ca^{2+} activation of striated muscle. For example, it has been shown that slow fibres have contractile characteristics which are clearly distinct from those of fast fibres.

Slow fibres have a relatively high Ca^{2+} sensitivity and a relatively shallow T/pCa relationship, whereas fast fibres are less sensitive to Ca^{2+} and have very steep T/pCa relationships. This has been shown in rat (Stephenson & Forrest, 1980; Stephenson & Williams, 1981, 1982; Mizusawa et al., 1982; Fink et al., 1986; Eddinger & Moss, 1987; Lazewski-Williams et al., 1989; Danieli-Betto et al., 1990) guinea-pig (Takagi & Endo, 1977) rabbit (Kerrick et al., 1976; Donaldson, 1984; Metzger & Moss, 1987; Greaser et al., 1988; Gardetto et al., 1989) chicken (Reiser et al., 1987a) and human muscle fibres (Ruff, 1989). Similar differences in Ca^{2+} sensitivity have also been found between ventricular (slow) and atrial (fast) skinned fibres (Morano et al., 1988).

The Ca^{2+} sensitivity differences are mainly attributed to the different composition of the regulatory proteins. Whereas the steeper T/pCa relationship in fast fibres is indicative of a greater co-operativity in Ca^{2+} activation of tension compared to slow fibres. Although the exact mechanism of this effect is unknown, it is thought to be at least in part due to differences in the Ca^{2+} binding characteristics of the slow and fast isoforms of troponin and to the effect of neighbouring cross bridges (Metzger & Moss, 1987; Bremel & Weber, 1972).

However, certain investigators have observed different results to those described above (e.g., Moss et al., 1986; Mounier et al., 1989) but these have been explained on the basis of different temperature and SL (see below) in these latter studies.

The Ca^{2+} sensitivity differences could be explained by the studies of Stephenson & Williams (1981, 1985). They demonstrated that Ca^{2+} sensitivity decreases when temperature increases from 5 to 25 °C and that the respective position of the T/pCa relationship of both slow and fast muscles changes with temperature, i.e. increases in temperature shift the T/pCa relationship to higher Ca^{2+} concentration. The most likely explanation for this observation is that the affinity of the Ca^{2+} specific sites of Tn C for Ca^{2+} decreases with increasing temperature, as expected, since Ca^{2+} binding is an exothermic reaction, (Potter et al., 1977). However, it appears from their studies that whatever the temperature there was a lower Ca^{2+} threshold and a less steep T/pCa relationship in slow than in fast fibres.

In addition the effect of SL could be implicated in the differences in Ca^{2+} sensitivity. It has been reported by Stephenson & Williams, 1982 that an increase in SL (within the range 2.2 – 3.6 μm) shifted the T/pCa relationship for both slow and fast fibres towards higher pCa without affecting the steepness of the relationship. This result is consistent with the idea that the sensitivity of the contractile apparatus to Ca^{2+} is increased with increasing SL.

MUSCLE FATIGUE

Muscle fatigue, defined as loss of tension and power, probably results from a multiple of factors. Alterations in the functional capacity of the central nervous system (central fatigue), the peripheral α-motor nerves, the neuromuscular junction, and the skeletal muscles could all contribute to fatigue. However, evidence suggests that muscle fatigue is generally confined to the peripheral skeletal muscle cells (for review see Jones & Round, 1990).

Peripheral fatigue involves at least two components: disruption of excitation-contraction coupling and a change in the composition of the myoplasm which directly modulates cross bridge function. The relative contribution of these two components depends upon factors such as the intensity and duration of the contractile activity, as well as the fibre type composition of the muscle under study (for review see Fitts & Metzger, 1988).

Many studies on skinned muscle fibres have shown that the products of the ATPase reaction can have marked effects on contractile function.

A decrease in pH leads to a decrease in maximal isometric tension and V_{max}, whilst the T/pCa relationship is shifted to the right (i.e., a decrease in the Ca^{2+} sensitivity) (Fabiato & Fabiato, 1978; Donaldson & Hermansen, 1978; Robertson & Kerrick, 1979; Donaldson, 1984; Luney & Godt, 1987; Cooke et al., 1988; Metzger & Moss, 1987, 1988, 1990a, b; Godt & Nosek, 1989). Moreover, H^+ ions have been reported to effect fast fibres more than slow fibres (Donaldson, 1984; Metzger & Moss, 1990a, b).

An increased concentration of P_i in the mM range has been shown to decrease the maximal isometric tension, whilst V_{max} has been

relatively unchanged (Hibberd et al., 1985; Cooke & Pate, 1985; Kwai, 1986; Webb et al., 1986; Nosek et al., 1987; Hoar et. al., 1987; Kwai et al., 1987; Chase & Kushmerick, 1988; Cooke et al., 1988; Pate & Cooke, 1989a; Godt & Nosek, 1989; Millar & Homsher, 1990; Martyn & Gordon, 1992). Furthermore, a rightward shift in the T/pCa relationship with increased concentration of P_i has been reported by many investigators (Brandt et al., 1982; Hoar et al., 1987; Godt & Nosek, 1989; Millar & Homsher, 1990; Martyn & Gordon 1992).

Generally the effect of increased concentrations of ADP is in the opposite direction compared to the effect of H^+ ions and P_i. Increased concentrations of ADP lead to an increase in the isometric tension and a leftward shift in the T/pCa relationship (Hoar et al., 1987; Luney & Godt, 1987; Cooke & Pate, 1985). Although, V_{max} is reduced in the presence of increased concentrations of ADP (Luney & Godt, 1987; Cooke & Pate, 1985; Fortune et al., 1989). However, the effects of ADP on the contractile apparatus are minimal relative to the effects of H^+ ions and P_i (Cooke et al., 1988; Godt & Nosek, 1989).

Finally, the individual results obtained for the three ATP hydrolysis products (ADP, P_i and H^+) result in a summated effect, when all three products are present together. Moreover, the summated effects of the three products are comparable with changes observed during fatigue (Chase & Kushmerick, 1988; Cooke et al., 1988; Godt & Nosek, 1989).

EFFECT OF THYROID STATUS ON SKELETAL MUSCLES

The abnormal plasma levels of thyroid hormones found in hyper- and hypothyroidism are often associated clinically with abnormalities of muscle function (Engel, 1972; McKeran et al., 1975). Reports on muscle function in hyperthyroidism mention fast fatigue, severe weakness and wasting of muscles (Grob,1963; Ramsay, 1974). With respect to hypothyroidism the most reported complaints are; stiffness of the muscles, slowness of movement, difficulty in initiating movement and weakness and wasting of the muscles (Astrom et al., 1961; Ramsay, 1974; Rao et al., 1980). Although the weakness in hypothyroidism may be less prominent in comparison with the hyperthyroid situation, the same muscle groups are affected (Ramsay, 1974). The symptoms of disturbed muscle function in hyper- or hypothyroidism disappear when the euthyroid state is restored (Grob, 1963; Ramsay, 1974).

It has been shown that hyperthyroidism changes contractile, biochemical and histochemical characteristics of slow muscles to those resembling fast muscles, whilst hypothyroidism results in opposite but less dramatic changes (McKeran et al., 1975; Winder et al., 1975, 1980; Ianuzzo et al., 1977; McCardle et al., 1977; Winder & Holloszy, 1977; Janssen et al., 1978; Courtright & Fitts, 1979; Ianuzzo et al., 1980; Johnston et al., 1980a, b, 1983; Fitts et al., 1980, 1984; Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nwoye & Mommaerts, 1981; Kim et al. 1982; Nicol & Maybee, 1982; Nwoye et al., 1982; Hall-Craggs et al., 1983; Johnston & Turnbull, 1984; Sickles et al., 1987). The changes observed are quantitatively greater in muscles composed mostly of slow muscle fibres such as the rat soleus in comparison to fast muscles such as the EDL (Winder & Holloszy, 1977; Janssen et al., 1978; Ianuzzo et al., 1980; Winder et al., 1980; Johnston et al., 1980b Nicol & Bruce, 1981;

Nicol & Johnston, 1981; Nwoye & Mommaerts, 1981; Kim et al. 1982; Nicol & Maybee, 1982; Johnston & Turnbull, 1984; Fitts et al., 1984; Leijendekker & Hardveld, 1987; Sickles et al,, 1987; Fitzsimons et al., 1990).

The differential effects of thyroid hormone on slow and fast muscles could be due to a greater blood supply and capillary density in slow than in fast muscles and hence differences in hormone delivery (Janssen et al., 1979; Sillau, 1985a, b). Therefore, the effective concentration of the hormone in the slow muscle would be greater. However, this factor cannot account for results obtained in hypothyroid animals (e.g., Nwoye & Mommaerts, 1981; Fitzsimons et al., 1990). Alternatively, slow muscle has a greater range for potential change than does fast muscle.

However, the most likely explanation is that it is widely believed that the biological effects of thyroid hormone result from its binding to nuclear receptors that interact with chromatin and thereby alter gene expression (Schwartz & Oppenheimer, 1978; Russell et al., 1988) and it has been shown that the T_3 (triidothyronine) and T_4 (thyroxin) uptake is greater in slow than in fast muscles (Janssen et al., 1979). This suggests a quantitative difference in receptor number in muscle cells, slow muscles containing more that fast. In fact, it has been shown that tissues with a significant response to thyroid hormones contain a larger number of nuclear receptor sites than do unresponsive tissues (Oppenheimer, 1975). Therefore, it is possible that the differential effects are due to either different levels of receptor or different levels of responsiveness of the receptor to thyroid hormone among the different muscle types. A higher receptor concentration in the slow muscle would explain its greater responsiveness in both hyper- and hypothyroidism.

The mechanism through which the contractile, biochemical and histochemical characteristics are changed by thyroid hormones is under debate. Theoretically the changes could be the result of a direct effect of thyroid hormones on the muscle or an indirect effect of altering neuronal activity. Johnston et al. (1980b) prevented the effects of hypothyroidism by denervation of the muscle and therefore concluded the change was neurally mediated. However, these results could be alternatively explained by superimposed effects of denervation (shift to fast muscle) on the hypothyroid (shift to slow muscle) state. In fact, the hyperthyroid induced shift to fast muscle produced in rat soleus was not cancelled by denervation but rather accentuated (Nwoye et al., 1982; Hall-Craggs et al., 1983). Nwoye & Mommaerts (1981) also showed that fast to slow conversion of EDL muscle in hypothyroidism was accentuated and not cancelled by denervation. Therefore, it appears that thyroid hormone and denervation control muscle phenotype through different mechanisms (McCardle et al., 1977; Nwoye & Mommaerts, 1981; Nwoye et al., 1982; Johnston et al., 1983; Johnston & Turnbull, 1984).

In addition, Sickles et al. (1987) have showed that hyperthyroidism selectively increases the oxidative metabolism of the slow-oxidative motor units of soleus muscles. This could be either by a direct effect on the motorneurons themselves or merely a secondary effect resulting from the inter conversion of SO fibres.

EFFECT ON CONTRACTILE PROPERTIES

Hyperthyroidism shortens the contraction and relaxation time of both the isometric twitch and tetanus contractions in the rat soleus muscle (Table 1.3), with the effects on relaxation time being greater than contraction time (Gold et al., 1970; Takamori et al., 1971; Fitts et al., 1980, 1984; Nicol & Bruce, 1981; Nicol & Maybee, 1982). Hyperthyroidism also increases the rate of tension development and decline of tension during both twitch (Fitts et al., 1980, 1984; Nicol & Bruce, 1981; Nicol & Maybee, 1982) and tetanic contractions (Nicol & Bruce, 1981).

On the other hand, studies on the slow muscle of the hypothyroid rat have shown a prolongation in contraction and relaxation time (Gold et al., 1970; Takamori et al., 1971; Johnston et al., 1980a, b; Montgomery, 1984). Hypothyroidism also produces a marked slowing in the rate of contraction and relaxation of the soleus isometric twitch and a reduction in the maximum rate of tension development during a tetanus (Johnston et al., 1980a, b).

In fast muscle, the effects of thyroid hormone levels are much less pronounced. In hyperthyroidism the twitch duration is either unaffected (Fitts et al., 1984) or decreases slightly which is mainly due to a reduction in relaxation time (Nicol & Bruce, 1981). In a twitch contraction, the rate of tension development is also largely unaffected, whereas the rate of tension decline shows a progressive increase with thyroid hormone treatment from 1-6 weeks, though the effect is not significant at any stage of the treatment (Nicol & Bruce, 1981). In a tetanic contraction, the rate of tension development and decline has been shown to be unaffected (Fitts et al., 1984) or there is a slight increase in the rate of relaxation (Nicol & Bruce, 1981).

In hypothyroidism, the relaxation time of the EDL was prolonged during a twitch and tetanus contraction whilst contraction time was unchanged during a tetanus (Leijendekker & Hardveld, 1987). However, Johnston et al. (1980b) found the twitch characteristics of EDL muscle to be unchanged with hypothyroidism.

The increased rate of relaxation in hyperthyroid soleus muscles can probably be attributed to a faster Ca^{2+} removal from the cytoplasm and as such thyroxin treatment has shown an increased rate of Ca^{2+} uptake by the SR (Fitts et al., 1980; Kim et al., 1982; Nwoye et al., 1982) and an induced proliferation of the SR (Fitts et al., 1980; Kim et al., 1982). The two-fold increase in the rate of tension decline following a tetanic contraction correlates well with the two-fold increase in SR content in the soleus (Fitts et al., 1984; Kim et al., 1982). In addition parvalbumin, a high affinity Ca^{2+} binding protein which may facilitate the rate of muscle relaxation in euthyroid muscles, could be involved in faster relaxation in hyperthyroidism (Heizmann, 1984; Gillis, 1985). However, Muntener et al. (1987) found that parvalbumin distribution and concentration was largely unaffected in all thyroid states.

The increased rate of contraction in the hyperthyroid soleus muscles can be explained by the same mechanism, in that induced adaptations in the SR might result in a more rapid increase in cytoplasmic Ca^{2+} during E-C coupling, i.e., a faster rate of Ca^{2+} diffusion from the more extensive SR network. However, this would only hold, if the rate at which Ca^{2+} is made available to troponin limits this phase of contraction and there is considerable uncertainty regarding whether the rate at which Ca^{2+} is made available to Tn limits the rate of actin-myosin cross bridge activation (Julian & Moss, 1976). Moreover, a faster rate of contraction could partially be explained by the less elastic nature

of the hyperthyroid soleus muscle (Nicol & Maybee, 1982) because muscles with a less extensible series elastic component have faster rates of tension production (Julian & Moss, 1976).

In fast muscle, the observation of little or no change of contraction and relaxation rate in hyperthyroidism is consistent with thyroid hormone treatment having no effect on the yield (mg/g) or specific activity of the SR ATPase enzyme in fast muscle (Kim et al., 1982). In addition, there was no change observed in the elasticity of the fast EDL muscle in hyperthyroidism (Nicol & Maybee, 1982).

Conflicting results have been obtained for isometric tetanic tension (Table 1.3). In slow muscle, tetanic tension appears to be relatively unaltered by hyperthyroidism (Fitts et al., 1980, 1984) or it has been observed to increase by 20% after 6 weeks of hormone treatment (Nicol & Bruce, 1981). On the other hand in fast muscle, in both hypo- and hyperthyroid muscles, tetanic tension has been shown to be unchanged (Nicol & Bruce, 1981; Fitts et al., 1980, 1984; Leijendekker & Hardveld, 1987).

Conflicting results have also been obtained for isometric twitch tension (Table 1.3). In slow muscle, some investigators have found isometric tension to increase by 30–60% after 2–6 weeks of T_3 treatment (Nicol & Bruce, 1981), whilst others have found it to decrease by 25% after 6 weeks of T_3 treatment (Gold et al, 1970; Fitts et al., 1980, 1984). On the other hand, Everts, 1983 found no difference in the isometric twitch tension of the soleus muscle between the three different thyroid states and Gold et al., 1970 found no difference between hyper- and euthyroid soleus muscles.

In fast muscle, hyperthyroidism has resulted in no significant effect on isometric twitch tension (Nicol & Bruce, 1981; Fitts et al.,

1984) or it has decreased by 19% (Everts, 1983) with respect to the euthyroid state. Whilst hypothyroidism has produced an increase of 12–15% in isometric twitch tension (Everts, 1983; Leijendekker & Hardveld, 1987) with respect to the euthyroid state. One study on the triceps surae muscle (gastrocnemius-plantaris-soleus muscle group) which is a combination of slow and fast muscles has shown an interesting result in that the author observed a 14% increase in the hypothyroid state and a 9% decrease in the hyperthyroid state with respect to the euthyroid state (Everts, 1983).

Conflicting results have also been obtained with respect to V_{max} (Table 1.3). In slow (soleus) muscle, investigators (Nicol & Bruce, 1981) using an indirect measurement of V_{max} (i.e., using maximal rate of isometric tension as a valid measure of V_{max} – Close, 1972; Drachman & Johnston, 1973) have found an increase in the V_{max} of hyperthyroid soleus muscles versus (vs.) euthyroid. But whether this indirect measure is a valid measurement of V_{max} has been questioned by Fitts et al. (1980) who found a similar result as above but observed no change in V_{max} when measured directly. It is tacitly assumed that changes in contraction time reflect changes in the intrinsic speed of shortening, but this is not necessarily so. Changes in the contraction time of the twitch may be due to changes in the amount and/or kinetic properties of its SR (Drachman & Johnston, 1973; Ramirez & Pette, 1974) without any modification of the contractile proteins, whereas V_{max} of muscle is determined by the ATPase activity of its myosin (Barany, 1967). However, some investigators measuring V_{max} directly have observed an increase in the V_{max} of hyper- soleus muscle vs. hypothyroid muscle (Gold et al., 1970; Montgomery, 1984; Collings & Montgomery, 1990). Whilst, others have found no change (Fitts et al., 1980, 1984).

In fast (EDL) muscle, little information exists, but Fitts et al. (1984) found hyperthyroidism to have no effect on V_{max} of EDL muscle and Leijendekker & Hardveld (1987) found hypothyroidism to have no effect either.

Table 1.3: Summary of contractile properties of hyper- and hypothyroid slow and fast muscles. The contractile measurement either decreases (dec), remains unchanged (uc) or (inc) increases with respect to the euthyroid or the opposite state. More than one entry shows the conflicting results obtained by different investigators.

	SLOW MUSCLE		FAST MUSCLE	
	Hyperthyroid	Hypothyroid	Hyperthyroid	Hypothyroid
Twitch tension	dec/uc/inc		dec/uc	inc
Contraction time	dec	inc		
Relaxation time	dec	inc	dec slightly	inc
Contraction rate	inc	dec	uc	
Relaxation rate	inc	dec	inc	
Tetanic tension	uc/inc		uc	uc
Contraction time	dec			uc
Relaxation time	dec			inc
Contraction rate	inc	dec	uc	
Relaxation rate	inc	uc	uc/inc	
V_{max}	uc/inc	dec	uc	uc

EFFECT ON MYOSIN AND MYOFIBRILLAR ATPase ACTIVITY

In the soleus muscle, the Ca^{2+} activated myosin ATPase activity has been reported to be decreased in the hypothyroid state, and increased in the hyperthyroid state of the rat (Janssen et al., 1978; Ianuzzo et al., 1977, 1980; Johnston et al., 1980a; Nwoye et al., 1982). Furthermore, Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity has been observed to increase in the hyperthyroid state, and decrease in the hypothyroid state of the soleus muscle (Janssen et al., 1978; Nwoye et al., 1982).

On the other hand, Fitts et al., (1980) have reported no change in the Mg^{2+} activated actomyosin ATPase activity in hyper- compared to euthyroid soleus muscles. Moreover, a recent study, by Fitzsimons et al. (1990) found no difference in the Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity between hyper- and hypothyroid soleus muscles.

In fast muscles of the rat, there is conflicting evidence with respect to a decrease or increase in the Ca^{2+} activated myosin ATPase activity due to hypo- and hyperthyroidism respectively. Ianuzzo et al. (1980) found the Ca^{2+} activated myosin ATPase activity of the fast plantaris muscle relatively unchanged in hyper- compared to hypothyroid muscles. Whereas, the Ca^{2+} activated myosin ATPase activity in the fast EDL muscle has been shown to increase 34% (significant) in hyper- compared to hypothyroid muscles (Nwoye & Mommaerts, 1981). Although, it must be borne in mind that, the Ca^{2+} activated myosin ATPase activity was found to be decreased in the hypothyroid EDL muscle and to be unchanged in the hyperthyroid EDL muscle of the rat when comparing with the euthyroid muscle (Nwoye & Mommaerts, 1981).

Furthermore, results obtained by Leijendekker & Hardveld (1987) seem to be in an intermediate position. They have shown that the Ca^{2+} activated myosin ATPase activity was not significantly affected in hypothyroid gastrocnemius muscles, although the mean values tended to be somewhat lower than euthyroid muscles.

A similar kind of scenario exists when the myofibrillar ATPase activity is measured, for example, the Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity of the (white) gastrocnemius muscle is relatively unaffected by dysthyreosis (Leijendekker & Hardveld, 1987; Fitzsimons et al., 1990). Whereas, measurements of Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity in the (red) gastrocnemius muscle have shown a 53% (significant) increase in the Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity of hyperthyroid muscles compared to hypothyroid muscles (Fitzsimons et al., 1990).

EFFECT ON MYOSIN HEAVY AND LIGHT CHAINS

In the soleus, hyperthyroidism decreases the expression of type I skeletal MHC gene, increases the expression of type IIA MHC gene and newly induces the expression of the type IIB MHC gene (Gustafsson et al., 1985; Izumo et al., 1986). Hypothyroidism, on the other hand, deinduces the expression of the type IIA MHC gene to undetectable levels, and newly induces the expression of the embryonic MHC gene, whilst it does not change the level of expression of the type I MHC gene, which is already high in euthyroid soleus muscles. (Izumo et al., 1986; Diffee et al., 1991).

In fast muscles, hyperthyroidism deinduces the type IIA MHC gene to undetectable levels, whilst leaving the expression of the type IIB MHC gene unchanged, which is already high in euthyroid fast muscles. On the other hand, hypothyroidism increases the expression of the type I MHC

gene and leaves the expression of the type IIB MHC unchanged or decreases it slightly. It also slightly increases the expression of the type IIA MHC gene or newly induces it in a muscle such as the TFL (Izumo et al., 1986; Diffee et al., 1991).

Although, the existence or presence of a particular mRNA species does not necessarily indicate a priori that the specific protein product will be expressed. It has been shown that when comparing the distribution of the MHC mRNA level with the MHC isoforms in a given muscle a similar kind of distribution results (Diffee et al., 1991).

In this respect, in slow muscles, hypothyroidism generally appears to induce an increase in type I MHC content while decreasing type IIA and IIB MHC content. However, it must be borne in mind that the soleus muscle had no type IIB MHC (Diffee et al., 1991).

In fast muscle, hypothyroidism slightly, increases type I MHC primarily at the expense of a reduction in IIB MHC (Leijendekker & Hardveld, 1987; Diffee et al., 1991).

Furthermore, qualitative changes have also been observed when studying the distribution of myosin light chain isoforms.

In slow muscles, hyperthyroidism shifts the profile of the myosin light chains from slow to fast. In this respect, it increases the levels of MLC_{1f} and MLC_{2f} and also slightly increases the level of MLC_{3f} (Ianuzzo et al. 1980; Nwoye et al., 1982). Hypothyroidism, on the other hand, has generally the opposite effect. Hypothyroid soleus muscles are primarily composed of MLC_{1s} and MLC_{2s}, with a small percentage (9%) of fast light chains (MLC_{1f}, MLC_{2f} and MLC_{3f}) (Nwoye et al., 1982; Fitzsimons et al., 1990) or there is a complete disappearance of the MLC_{2f} (Johnson et al., 1980a, b).

In a fast muscle containing a majority of FOG fibres (e.g., red gastrocnemius), hyperthyroidism slightly reduces the level of MLC_{1s} and MLC_{2s}. Hypothyroidism, on the other hand increases the levels of MLC_{1s} and MLC_{2s} in conjunction with decreased levels of MLC_{1f}, MLC_{2f} and MLC_{3f} (Nwoye & Mommaerts, 1981; Fitzsimons et al., 1990).

In a fast muscle containing a majority of FG fibres (e.g., white gastrocnemius) the myosin light chain pattern is largely unaffected by dysthyreosis (Fitzsimons et al., 1990), or there is a slight increase in the level of MLC_{1f} in hyperthyroid muscles and a slight increase in the level of MLC_{1s} in hypothyroid muscles (Ianuzzo et al., 1980)

In addition, Fitzsimons et al. (1990) demonstrated that, in slow muscles, hyperthyroidism was associated with an increase in the relative amount of fast native isomyosin, whereas hypo- resulted in a relative increase in the slow native isomyosin content. Moreover, slow muscle was found to be more responsive to alterations in thyroid state than fast muscle.

In short, in slow muscle hyperthyroidism shifts the pattern of the MHC mRNA levels, native isomyosin isoforms, MHC and MLC isoforms from slow to fast whilst hypothyroidism results in opposite effects. Although, a similar kind of pattern is observed in fast muscle it is not as prominent.

EFFECT ON FIBRE POPULATIONS

In the rat, the thyroid hormone induced alterations in the fibre composition of skeletal muscle can be studied in detail, since one fibre type may predominate in a certain muscle, e.g., the soleus muscle contains predominantly slow-twitch motor units and 84% of the total fibre number are of the SO type, there are only 16% fast twitch fibres of the FOG type. The EDL, on the other hand is almost entirely composed of fast-twitch motor units, 97% of the fibres are of the fast twitch type (59% FOG and 38% FG) and only 3% are slow. Moreover, the TFL muscle is composed entirely of fast-twitch motor units, with 94% of the fibres of the FG type and 6% of the FOG type (Ariano et al., 1973).

There is general agreement that in the rat soleus, which has been used to represent a slow muscle by many investigators, the number of FOG fibres increases and decreases in hyper- and hypothyroid animals respectively at the expense of a decrease or increase in the SO fibres from the euthyroid state (Ianuzzo et al., 1977; Johnston et al., 1980a; Fitts et al., 1980; Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nicol & Maybee, 1982; Nwoye & Mommaerts, 1981; Nwoye et al., 1982). For example, in hyperthyroid muscles, the FOG fibres increase from 12–25% to 25–65%, due to a decrease of 74–88% to 35–73% in SO fibres. In hypothyroid muscles, the FOG fibres decrease from 12–25% to 0–8%, with an increase of 74–88% to 89–100% in SO fibres (Table 1.4). The range of values exhibited differ widely as a result of different degrees of induced hyper- and hypothyroidism, age and strain differences of the animals.

Table 1.4: Effect of dysthyreosis on fibre populations of rat soleus muscles.

Fibre type	Hyperthyroid	Euthyroid	Hypothyroid
SO	35–73%	74–88%	89–100%
FOG	25–65%	12–25%	0–8%

In fast muscle (the majority of investigators have used the EDL to represent a fast twitch muscle) the histochemical results in the hyperthyroid state have shown, the number of FOG fibres to increase mainly due to the conversion of FG fibres to FOG fibres, although the small number of SO fibres also get transformed. Whereas in the hypothyroid state, the SO fibres increase mainly due to the conversion of FOG fibres from the euthyroid state (Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nwoye & Mommaerts, 1981; Nwoye et al., 1982; Johnston et al., 1983). For example, in hyperthyroid muscles, the FOG fibres increase from 55–60% to 62–73%, due to a decrease of 34–44% to 25–40% in FG fibres and a small decrease in SO fibres from 1–4% to 0–1%. In hypothyroid muscles, the SO fibres increase from 4% to 19%, due to a decrease of 60% to 43% in FOG fibres and whereas the FG fibres remain unchanged at 37–38% (Table 1.5).

Table 1.5: Effect of dysthyreosis on fibre populations of rat EDL muscles.

Fibre type	Hyperthyroid	Euthyroid	Hypothyroid
SO	0–1%	1–4%	19%
FOG	62–73%	55–60%	43%
FG	25–40%	34–44%	37–38%

One study (Sickles et al., 1987), of the effect of hyperthyroidism on TFL muscles has shown no change in the fibre composition of this muscle. Control muscles possessed 95% FG and 5% FOG fibres whereas hyperthyroid muscles possessed 94.4% FG and 5.6% FOG fibres.

Finally, it must be noticed that the animal studies concerning the effects of thyroid hormone on the fibre composition of skeletal muscle do not mention significant alterations until 4 weeks with thyroid hormone (Nicol & Bruce, 1981). This time delay is consistent with the results of Gustafsson et al. (1985) who found a delayed onset of thyroid hormone action on the FOG MHC gene from soleus muscles. In addition, dysthyreosis has no effect on total number of fibres in either slow or fast muscle (Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nicol & Maybee, 1982).

It has been suggested that histochemical changes are not necessarily accompanied by alterations in shortening velocity. Fitts et al., (1980, 1984) found increased number of fast fibres in hyperthyroid soleus muscles but no change in the shortening velocity.

This discrepancy of an increased number of fast fibres in hyperthyroid soleus muscles without a change in V_{max} observed by Fitts et al. (1980) was explained on the basis that two types of light chains are found in myosin from fast twitch muscle, LC_{1f} and LC_{3f}. Both chains confer alkali stability but LC_{3f} has an *in vitro* ATPase activity which is twice that of LC_{1f} (Wagner & Weeds, 1977; Gauthier et al., 1978). Therefore, the suggestion is that the histochemical result is a reflection of the presence of LC_{1f}, which is known to increase in hyper- soleus muscles (Nwoye et al., 1982). However, later work at higher ionic strength showed no difference in the ATPase activity between the two different alkali light chains (Wagner et al., 1979; Wagner & Giniger,

1981).

Moreover, this same discrepancy of increased number of FG fibres but unchanged V_{max} in fast muscle was explained by Fitts et al. (1984), by assuming that the FG and FOG fibres have similar V_{max} and since there is mainly inter conversion between the two types of fast fibres then the histochemical profile of the muscle could be changed without a change in shortening velocity. However, work on single fibres has shown that FG fibres have higher shortening velocities than FOG fibres (e.g., Reiser et al., 1985a, b).

EFFECT ON MUSCLE FATIGUE

It is well documented that hyperthyroidism increases mitochondrial number and the respiratory capacity of hind limb muscle mitochondria (Gustafsson et al., 1965; Winder & Holloszy, 1977; Baldwin et al., 1978; Janssen et al., 1978; Nicol & Johnston, 1981) whereas hypothyroidism results in the opposite effect (Baldwin et al., 1978; Janssen et al., 1978; Nicol & Johnston, 1981).

Increases in the mitochondrial number and respiratory capacity have been observed following a program of exercise training (Holloszy & Booth, 1976) where the effect has been associated with an enhanced resistance to fatigue (Fitts et al, 1980). Whether or not changes with dysthyreosis have functional importance in the onset of fatigue is unknown. However, one study (Fitts et al., 1984) showed that hyperthyroidism delayed the onset of fatigue in the soleus whilst there was no effect on the EDL muscle.

The metabolic advantage of the oxidative fibres in these muscles is lost in the skinned fibre preparation, thus any differences observed in conditions mimicking fatigue will be due to the regulatory and contractile proteins. To date no single fibre studies have been reported on the effects of dysthyreosis on contractile function under conditions mimicking fatigue.

SKINNED FIBRE PREPARATION

Skinned muscle fibres, (i.e., fibres from which the surface membrane has been removed or made highly permeable by membrane skinning) present a very promising approach to many problems of muscle. They allow the composition of the myofibrillar space to be altered whilst the structural organisation of the myofibrils remains relatively physiological. Therefore, they have come to be widely used in studies on the mechanisms of contraction.

There are two main techniques for removing the sarcolemma, either mechanical or chemical (for review see Stephenson, 1981). The mechanical techniques involve micro-dissection of the sarcolemma under inert oil (Natori, 1954), splitting of the fibre into two, each coated on one side by the intact sarcolemma (Endo, 1977) and the disruption of the sarcolemma by carefully controlled homogenisation (Fabiato & Fabiato, 1972). This latter process is mainly used for skinning cardiac cells and fibres prepared this way have their SR intact.

The chemical methods involve rendering the sarcolemma highly permeable by chemical treatment. The various methods include treating fibres with glycerol (Julian, 1971), a non-ionic detergent such as Brij 58 (polyoxyethylene–20–cetyl ether) (Simmons & Szent-Gyorgyi, 1978) or triton X-100 (Miller et al., 1985) or chelating agents such as EDTA (ethylenediamine tetra acetic acid) and EGTA (ethylene glycol tetra acetic acid) (Miller, 1979).

Irrespective of the manner of preparation, the contractile structure of the myofilaments after skinning is accessible to and may be influenced by the free Ca^{2+} concentration of the bathing medium. The latter must of course, contain ATP as an energy source since skinned fibres are unable to synthesise it in the absence of substrates. ATP-containing bathing

medium should also resemble the myoplasm as closely as possible since pH, Mg^{2+} concentration, ionic strength and the temperature all affect the responsiveness of the contractile structure to Ca^{2+} (Ashley & Moisescu, 1977; Godt & Lindley, 1982).

Another advantage of the skinned fibre preparation is that the direct effect of metabolites such as H^+, and P_i on the contractile system can easily be assessed. Whilst the removal of the SR avoids the complications associated with calcium movements. Such experiments would be impossible to perform on live fibres because of diffusion problems and the inability to control or measure variables easily.

SCOPE OF THE STUDY

The role of thyroid hormones in modifying the characteristics of adult muscle fibres has received considerably less attention than that of neural factors. Therefore, this study will principally focus on the effect of triidothyronine on skeletal muscles.

The aims and objectives of the study were as follows:

1) To characterise the values of chemically skinned single muscle fibres from soleus and TFL muscles with respect to maximal isometric tension and V_{max} before embarking on studies with treated animals. However, in view of the contradictory results reported (e.g., Metzger & Moss, 1987; Mounier et al., 1989) for the tension generated by slow and fast muscles/fibres, the study will try to compare the tension generated by single fibres from slow (soleus) and fast (TFL) twitch muscles of the rat and Guinea-pig. The approach of using single fibres eliminates the inherent problems of interpreting results from studies on whole muscles which usually contain heterogeneous fibre types with respect to contractile properties.

Additionally, many investigators (Moss et al., 1982; Reiser et al., 1985a, b; Metzger & Moss, 1987, 1990; Godt & Nosek, 1989; Mounier et al., 1989) when using single skinned fibres to measure tension and/or V_{max}, have initially stored bundles of fibres in glycerol for upto 5 or 6 weeks before use. This aids in analysis of more fibres in one muscle and thus leads to use of less animals. However, no study is known to have studied the short and long term effects of glycerol storage on the values of maximal tension and V_{max}, therefore, a second aim was to ascertain whether these variables are affected in any manner. Before undertaking these experiments the optimum operating temperature and sarcomere length were determined.

2) Firstly, it was to examine if changes in a particular mechanical property, V_{max} was changed in hyper- or hypothyroidism in skinned fibres from soleus and TFL muscles, secondly to examine if the isometric tension was changed in any manner and thirdly to correlate these changes, if any, with the histochemical typing of the single fibres. To this end a technique was developed where isometric tension, V_{max} and the histochemical reaction could be measured in the same fibre.

Since only mild forms of hyper- or hypothyroidism were administered the range of change induced was fairly narrow and this probably represents the extreme ends of the 'normal' spectrum, before either full blown hyper- or hypothyroidism results. Thus it is likely that the control animals will have thyroid hormone levels scattered throughout this range – though biased more towards the bottom end of the spectrum. Therefore, comparison of hypothyroid with control or control with hyperthyroid may not yield significant differences and any changes would be better highlighted between the two extreme states.

3) In view of the different characteristics of the T/pCa relationship from euthyroid slow and fast muscle fibres, the aim was to examine possible changes in the T/pCa relationship generated by single skinned fibres from soleus and TFL muscles of hyper- and hypothyroid animals. This would be indicative of any changes in the Ca^{2+} regulatory proteins in the same type of fibre, as identified histochemically, between the two treated states.

Furthermore, if the main effect of hyperthyroidism is to change a slow muscle into a 'fast' muscle and vice versa in hypothyroidism, then the T/pCa relationship of the hyperthyroid soleus fibres would be expected to have characteristics which are more akin to fast fibres, whereas the T/pCa relationship of hypothyroid soleus fibres would be

more akin to 'slow(er)' fibres.

4) Considerable alterations in intracellular milieu are observed in fatigued muscle (e.g. Dawson et al., 1980) and certain of these are known to directly affect the contractile apparatus (e.g. the decline in pH and rise in P_i, Fabiato & Fabiato, 1978; Brandt et al., 1982).

Therefore, one of the aims was to examine the possible effects of a decreased pH (pH 6.6) or an increased concentration of P_i (7.5 mM) on the Ca^{2+} sensitivity (T/pCa relationship), maximum isometric tension and V_{max} of hypo- and hyperthyroid soleus and TFL muscle fibres. An intracellular pH of 6.6 is known to occur in intact fibres during exercise to the point of fatigue (Hermansen, 1981). Whereas 7.5mM P_i has been shown to modify the T/pCa relationship and isometric tension (Brandt et al., 1982).

Furthermore, it has been shown that euthyroid fast fibres are more sensitive to decreased pH than euthyroid slow fibres with respect to contractile dysfunction (Donaldson, 1984; Metzger & Moss, 1987, 1988). Whereas at present, there is no reported differential effect of P_i between slow and fast muscles/fibres. Thus, another aim was to see if the reported differential effect of decreased pH existed between the two thyroid states within each muscle type, and if there was any differential effect of P_i on the soleus and TFL muscle fibres from hyper- and hypothyroid animals.

5) Firstly to measure the Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity of hypo- and hyperthyroid soleus and TFL muscles, and to determine whether there was any difference between the two different thyroid states within each muscle type. Secondly, to determine the fibre populations of each of the four groups (viz., hypo-soleus, hyper- soleus, hypo- TFL and hypo- TFL muscles).

Thirdly, it was to determine whether there was any correlation between the myofibrillar ATPase activity and the number of fast fibres (as determined histochemically) present in a muscle irrespective of thyroid status. This is because the practice of classifying slow and fast twitch fibres by the intensity of the histochemical staining reaction for myosin ATPase is based upon the assumption that the histochemical staining reaction accurately reflects the biochemical properties of the myosin which make it fast or slow. While this relationship appears valid for normal adult muscle (Guth & Samaha, 1969), it may not necessarily hold in muscles from hyper- and hypothyroid animals, as suggested by Fitts et al. (1980).

METHODS

ANIMALS AND TREATMENTS

Male Wistar rats with initial body weights of 230 – 270g and of similar ages (6 – 8 weeks old) were housed three to a cage at a room temperature of 18 °C - 20 °C on a 12 hour lights on 12 hour lights off schedule. All the animals were provided with food (rat and mouse modified maintenance diet BP 801155 W) and water ad libitum.

Three groups of male Wistar rats were used, viz.

a) Hyperthyroid b) Euthyroid c) Hypothyroid

Thyroid hormone treated animals were injected intraperitoneally for six weeks with 400 ug/Kg of Liothyronine sodium B.P. (T_3), dissolved in 0.9% NaCl made alkaline with 1 M KOH and adjusted to pH 8.5 with HCl, every second day due to T_3 having a half-life of 2 days. The dose of T_3 used had previously shown an increase in the level of circulating T_3, by 2-3 times, from 0.996 to 2.979 ng/ml, after a treatment period of six weeks without any significant changes in normal growth or development of the experimental animals (Nicol & Bruce, 1981; Nicol & Johnston, 1981). Euthyroid rats were not housed together, but picked at random from stock animals with a weight and age range similar to the treated animals. Euthyroid animals were not injected with saline, as this had previously been found to be unnecessary (Nicol & Johnston, 1981). Hypothyroidism was induced by dosing the animals for six weeks with carbimazole/methimazole (70 mg/L) via the drinking water used by the animals. The dose selected was to try to ensure that the animals gained weight normally, i.e. the dose gave a lowered thyroid hormone level which was, however, at the lowest end of the physiological range, i.e., not pathologically hypothyroid (Nicol & Johnston, 1981).

Euthyroid guinea-pigs were kept in similar conditions as above and given food and water ad libitum.

MUSCLE SAMPLES

The animals were weighed prior to death and killed individually, by a blow to the head. Immediately after death, the core temperature of treated animals was taken by inserting a thermometer into the rectum. Thereafter, the soleus and/or Tensor Fascia Latae (TFL) muscles from both hind limbs were dissected free from fat, connective tissue and excess tendon.

HISTOCHEMISTRY

The muscles were dissected immediately after the death of the animal and transverse sections of 2 - 4 mm thick were removed from the middle section of each muscle. Transverse 10 μm serial sections were cut and transferred onto coverslips. The coverslips were air dried on ice for at least 1 hour before being stained for the activities of succinic dehydrogenase (SDH) and for myosin ATPase (adenosine triphosphatase) after acid pre-incubation and neutral fixation. The histochemical stains produced by these enzymes were used for the classification of the fibre types in the soleus and TFL muscles.

Succinic dehydrogenase (SDH) activity

SDH is an oxidative enzyme localised in the mitochondria. It catalyses the reaction of succinate to fumarate. The corresponding release of electrons reduces the nitroblue tetrozolium salt to the insoluble diformazan salt which become localised as purple crystals at the site of enzyme activity. The number of electrons released and hence the intensity of the colouration achieved is a measure of the oxidative capacity of the fibre. Fibres that are highly oxidative, such as FOG fibres, have areas of dark purple localised under the sarcolemma and can be distinguished from SO and FG fibres which have lighter and no areas of purple stain respectively.

SDH activity was demonstrated by the method of Nachlas et al. (1957). Sections were immersed in Columbia jars containing 100 mM phosphate buffer, pH 7.6, with 70 mM sodium succinate and 1 – 2 mg/ml Dinitrotetrazolium blue (added just prior to the start of the incubation). Incubation was carried out in the dark at 37 °C for 40 minutes. The sections were then thoroughly washed for 30 - 60 minutes in distilled H_2O before being dried and mounted, using a drop of warm glycerol-

gelatin, onto microscope slides.

Myosin ATPase activity

One of the most important properties of myosin ATPase is its ability to hydrolyse adenosine triphosphate (ATP) which provides the energy necessary for muscle contraction. Barany, 1967 showed that the speed of contraction of a muscle was directly proportional to its myosin ATPase activity. Therefore a fast fibre is going to have a faster rate of hydrolysis of ATP than a slow fibre and thus the amount of product (adenosine diphosphate + inorganic phosphate) formed for any given time will be greater in a fast fibre than a slow fibre. The amount of inorganic phosphate (P_i) liberated by this reaction is directly proportional to the number of ATP molecules hydrolysed, and hence to the activity of the muscle fibres. The amount of P_i formed may be visualised by converting it to the cobalt salt and converting this to the insoluble sulphide. The brown/black deposit formed may then be clearly seen under light microscopy.

To allow for more accurate fibre typing, acid pre-incubation and neutral fixation were employed. Due to the acid lability of the myosin ATPase found in fast fibres, acid pre-incubation inactivates fast fibres and only slow fibres are darkly stained, whilst the fast fibres are lightly stained by the subsequent ATPase reaction. Neutral fixation differentiates between slow and fast fibres because the myosin ATPase activity of slow fibres is slower than fast fibres, thus the fast fibres are darkly stained whilst the slow fibres are lightly stained.

The activity of myosin ATPase in the different fibre types was demonstrated by a modification of the method of Davies & Gunn (1972). The present method utilised an acid pre-incubation stage to exploit the well documented (Guth & Samaha, 1969; 1970) differential pH

sensitivities of myosin ATPase from fast and slow fibres. Neutral fixation was used instead of alkaline pre-incubation which results in a similar effect.

Acid pre-incubation

Sections were incubated in Columbia jars with 100 mM sodium acetate buffer, pH 4.3, for 10 minutes at room temperature. The sections were then placed in the myosin ATPase incubation medium described below.

Neutral fixation

Sections were fixed for 2 minutes at 4 °C in Columbia jars with 100 mM sodium cacodylate buffer, pH 7.0, containing 4% formaldehyde. After being quickly washed in incubation medium, the sections were transferred to fresh myosin ATPase incubation medium.

Myosin ATPase incubation medium

Acid pre-incubated or fixed sections were incubated at 37 °C for 40 minutes in Columbia jars with 200 mM Tris - HCl, pH 9.5, containing 18 mM $CaCl_2$ and 2.67 mM ATP. The sections were then very quickly rinsed once in distilled H_2O, placed for 2 minutes in 2% $CoCl_2$, rinsed once more in distilled H_2O and finally placed for 1 minute in 0.5% ammonium sulphide. After a thorough wash in distilled H_2O for 30 - 60 minutes the sections were dried and mounted, using a drop of warm glycerol-gelatin, onto microscope slides.

The slides were examined under a microscope and the fibres from a whole muscle or single fibres were classified as slow oxidative (SO), fast oxidative glycolytic (FOG) or fast glycolytic (FG) on the basis of staining reactions for SDH and myosin ATPase. SO fibres exhibit a low myosin ATPase activity and a moderate oxidative capacity; FOG fibres exhibit a high myosin ATPase activity and a high oxidative capacity; FG fibres exhibit a moderate to high myosin ATPase activity and a low oxidative capacity.

V_{max} AND ISOMETRIC TENSION

An intrinsic property of muscle is its ability to shorten when activated, and maximum speed of muscle shortening is one of the most useful mechanical parameters for characterisation of the various types of muscles.

There are two ways of measuring the maximum velocity at which a skeletal muscle preparation can shorten. It can be estimated by the traditional method of extrapolating the hyperbolic force-velocity relationship (Hill, 1938) to zero external load, V_o. Or it can be measured from the time it takes a fully activated muscle fibre to take up slack, V_{max} (Edman, 1979).

The traditional method to estimate the maximum speed of shortening, is to allow the muscle to shorten against constant loads (isotonic) or constant velocities (isovelocity) and determine the force-velocity characteristics of the muscle over a range of loads (Hill, 1938). V_o is then typically estimated by fitting the Hill equation to the force-velocity data by various means and then extrapolating this equation to zero load (Hill, 1938; Edman et al., 1976; Julian et al., 1986b). However, it has been shown (Edman et al., 1976; Clafin & Faulkner, 1989) that the fitted hyperbola results in less accurate estimation of the force-velocity relationship between measured data points at low and high loads (< 4% and > 80% of maximum isometric tetanic force).

As an alternative to V_o for characterising intrinsic shortening velocity, Edman (1979) introduced V_{max}, the velocity of unloaded shortening determined by the 'slack test'. The slack test is performed by applying a series of step releases of varying amplitudes to a fully activated muscle fibre and measuring the time required to take up the resultant slack. The fibre shortens under unloaded conditions over the

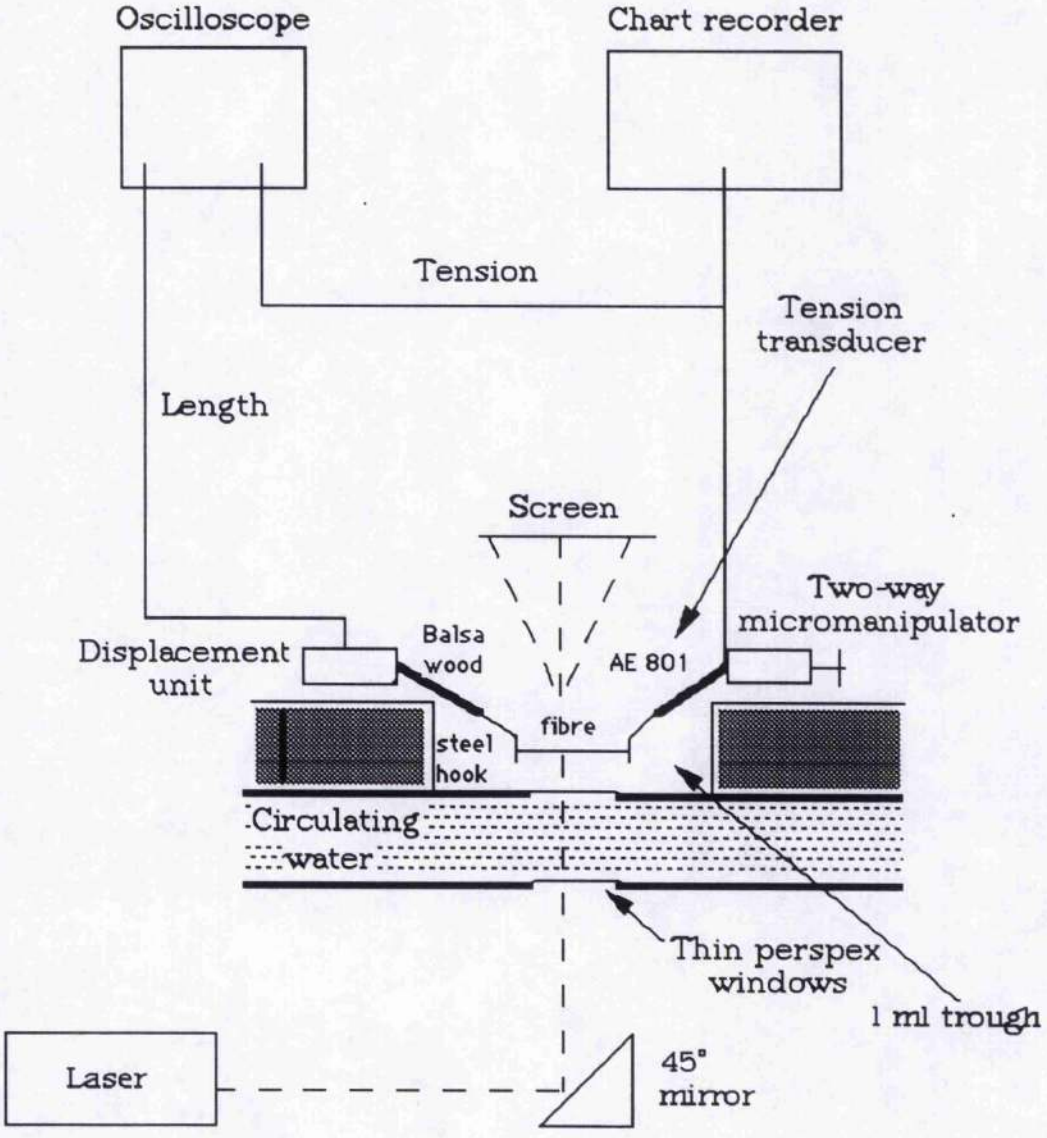
measured time interval if releases are of sufficient speed and amplitude, plotting the duration of the unloaded shortening intervals with release amplitudes yields a straight line. The slope of the line represents V_{max}, a direct measure of the unloaded shortening velocity of the fibre. Edman (1979) demonstrated that V_{max} is not different from V_o (at low and intermediate loads) in experiments where both velocities were determined for the same single skeletal muscle fibre. The slack test has been used subsequently to measure V_{max} of skinned single skeletal muscle fibres by many investigators (e.g., Julian & Moss, 1981; Metzger & Moss, 1988).

Due to the uncertainty of extrapolating to zero load and the deviation of the fitted hyperbola from experimental points at low and high loads in the traditional method, the slack test method (Edman, 1979) was employed to measure V_{max}. The apparatus used was similar to that described by Johnston & Sidell (1984).

DESIGN OF THE APPARATUS

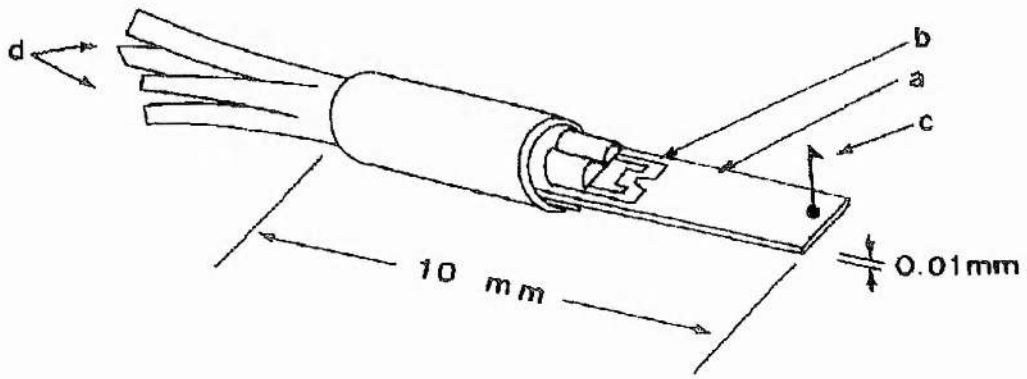
The experimental chamber was constructed from perspex and contained three water-jacketed troughs. The solutions were held in 1ml capacity troughs set into the temperature controlled perspex block. The fibre was transferred between experimental solutions contained in small troughs on a movable, spring-mounted holder. The temperature of the block was regulated by water which circulated through the chamber to maintain the temperature of the solutions. The water temperature in itself was controlled by circulating the water through a water bath. The temperature of the solutions was checked intermittently through the course of an experiment (Fig. 2.1).

Fig. 2.1: Diagram of apparatus used in the study for the measurement of isometric tension and maximum velocity of shortening. A microscope installed over the bath was used to measure the length and diameter of the fibre.



The tension measurement unit consisted of a stainless steel hook, attached (glued) directly to the silicon beam of an AE 801 strain gauge element (Fig. 2.2) average sensitivity 0.4 mNV^{-1} noise < 5 mV and drift < 1 mVh^{-1}. The element was held rigidly in a screened perspex adapter, and mounted on a two-way micro manipulator to allow for the adjustment of fibre length by adjusting the distance between the hooks. In this manner the sarcomere length (SL) of the fibre was set.

Fig. 2.2: Diagram of a silicon strain gauge tension semiconductor transducer (AE 800 series, Aksjeselskapet Mikro-Electronik, 3191 Horten, Norway). The silicon beam (a) has one diffused resistor (b) on each surface of the beam. Bending of the beam causes a change in the values of these resistors. The fibre is attached to hook (c). (d) leads.



The displacement measurement unit consisted of a stainless steel hook attached to a stout balsa wood beam 6 cm long and < 20 mg in weight glued to the centre of a loudspeaker coil from which most of the supporting core had been removed. The position of the beam was controlled by varying the amount of current passing through a 4 Ohms loudspeaker (Coil model RS01N6293). A flag of aluminium foil attached to the beam was arranged to interrupt a series of infra red photo diodes and emitters allowing the position of the beam to be monitored. A series of 10 pre-set 50 μm length steps could be applied to maximally activated fibres. A typical step release was achieved in < 1.5 ms.

The signals from the tension and displacement transducers were displayed on a storage oscilloscope (Hitachi V-134) and photographed on 35 mm film. Measurements from the negatives were made on a Carl Zeiss Jena (Germany) projector at magnification of 9 X from the screen of the oscilloscope to the projector screen. The tension was also monitored using a strip chart recorder (JJ instruments, Southampton) which had been calibrated with standard weights for a range of 0 - 200 mg. The calibration of the tension transducer was checked every week.

ISOLATION OF FIBRES

The dissected muscle was placed in a petri dish on ice and covered with paraffin oil. The muscle was pinned out and a small amount of cold relaxing solution (see next page) injected into the oil around the muscle, from this, small bundles of fibres were dissected under a microscope using light transmitted by optic fibres (avoids 'heating' of the fibres) and transferred onto a slide, with raised edges, containing a small amount of cold relaxing solution. From these bundles, single muscle fibres were dissected and transferred onto the stainless steel hooks using fine jeweller's forceps. Fibre segments with a final length of 1.5 - 2.5 mm in length were mounted horizontally in the apparatus by wrapping their ends around the two stainless steel hooks and secured using a drop of methacrylate polymer (perspex)/acetone glue to cover the part not involved in force generation (Altringham & Johnston, 1982). Each fibre was then immersed in the skinning solution. The thin covering of paraffin oil helped prevent dehydration during transference which was complete within 5 - 10 seconds.

SOLUTIONS

All the chemicals used in solution formation were sigma grade reagents. Three sets of solutions were used: Skinning, Relaxing and Activating. Relaxing solution was used as the basic solution from which the other two were modelled. It contained a buffer system (Piperazine-bis-2-ethane sulfonic acid – PIPES), a source of energy (ATP) a Mg^{2+} and Ca^{2+} buffer (Ethylene glycol tetra acetic acid – EGTA). Activating solution was made by the addition of $CaCl_2$ to the relaxing solution. Skinning solution contained 1% (w/v) polyoxyethylene-20-cetyl-ether (Brij 58), a non ionic detergent, in Relaxing solution. Brij 58 functionally destroys the cytoplasmic membrane and sarcoplasmic reticulum (Orientlicher et al., 1974). A rephosphorylating system (creatine kinase and creatine phosphate – CP) was used to maintain the MgATP concentration in the fibres. Creatine kinase in solid form was added to a final concentration of about 20 units/ml to the relaxing and activating solutions prior to each experiment.

The composition of all the solutions was determined with the aid of an iterative computer program (Nicol, 1985) which incorporated corrections for pH and temperature on stability constants. The ligand concentrations of all the solutions used were as follows:-

EGTA - 15 mM ATP – 6 mM CP - 15 mM PIPES - 20 mM

The concentration of Ca^{2+}, the ionic species and ionic strength of the various solutions used is summarised in Tables 2.1 – 2.8.

MEASUREMENT OF SARCOMERE LENGTH AND V_{max}

Sarcomere length was measured in relaxing solution after attachment of the fibre, by passing a beam of light from an He-Ne laser through the fibre. The diffraction pattern was viewed on a translucent screen placed at a fixed distance above the fibre. The sarcomere length was calculated using Bragg's equation for diffraction:

$$SL = \frac{\lambda}{\text{Sin } \theta}$$

where SL = sarcomere length, λ = wavelength of laser (0.6328 μm) and θ = angle subtended by the zero and first order diffraction patterns.

In the initial set of experiments sarcomere length was adjusted to between 3.0 - 3.1μm and then for subsequent experiments SL was adjusted to 2.7 - 2.8μm which corresponds to the optimum initial length for contraction in vivo in slow and fast muscles (Close, 1972). Fibre length (magnified 80 X) and diameter (magnified 160 X) were measured in situ using a graticule of a dissecting microscope. Tension in skinned fibres has been shown to be proportional to cross sectional area (e.g.: Hellam & Podolsky, 1969; Wise et al., 1971). Fibre cross-sectional area was calculated by equating fibre width to fibre diameter assuming a circular cross-section. The fibres were chemically skinned for > 10 mins and kept in relaxing solution for > 2 mins before activation, after activation they were returned to the relaxing solution.

V_{max} of individual fibres was determined by the slack-test method. Maximally activated (pCa 4.14) fibres were given a series of rapid releases of increasing magnitude such that each release caused a rapid fall in tension to zero and the tension remained at the zero level, as the fibre shortened to take up the slack. Since the fibre was still activated,

shortening occurred until the slack was taken up, at which point the tension began to redevelop. Following each release the fibre was re-extended to its original length. The time (T) was measured from the onset of release to the point (above the noise level) at which the tension started to redevelop. The amplitude of the release (L) was pre-determined to steps of 50 μm between 100 - 500 μm. V_{max} was determined from the slope of a plot of the applied length change, versus the time between the length change and the point when tension redeveloped (Plate. 2.1, Fig. 2.3 & 2.4). To express V_{max} in fibre lengths per second ($L_o\ S^{-1}$), the slope of the fitted line was divided by fibre length.

Plate 2.1: A typical photograph taken from the storage oscilloscope for measurement of V_{max}.

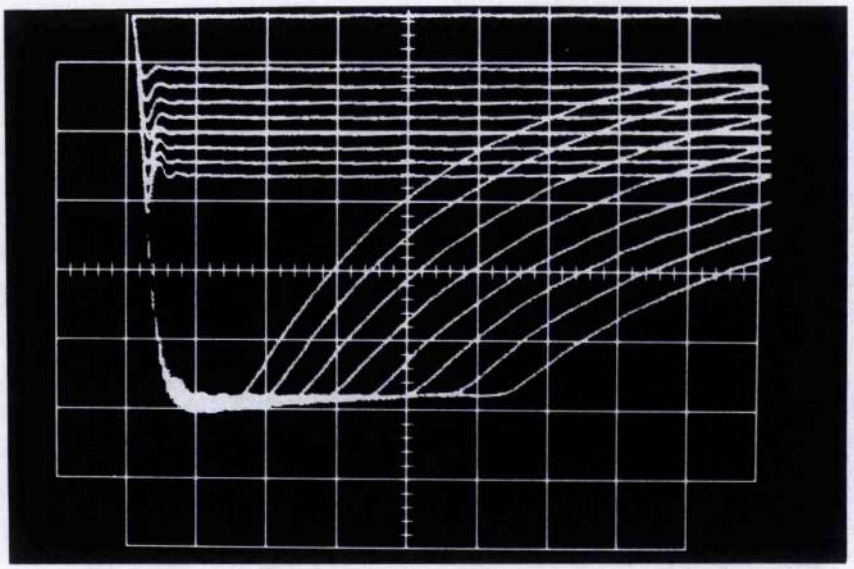


Fig. 2.3: A line diagram of plate 2.1, supplemented with the 'process' of measurement of V_{max}. V_{max} was determined from the slope of the applied length change (L_1, L_2 etc.), versus the time (T_1, T_2 etc.) between the length change and the point when tension redeveloped (P_1, P_2 etc.). The direction of shortening is downwards, tension redevelopment is upwards and the temperature was 25 °C. One problem with the slack test is the lack of definition of the point at which tension redevelopment starts as a result of noise. However, this can be overcome and more consistent results obtained, if a line is drawn above the noise level.

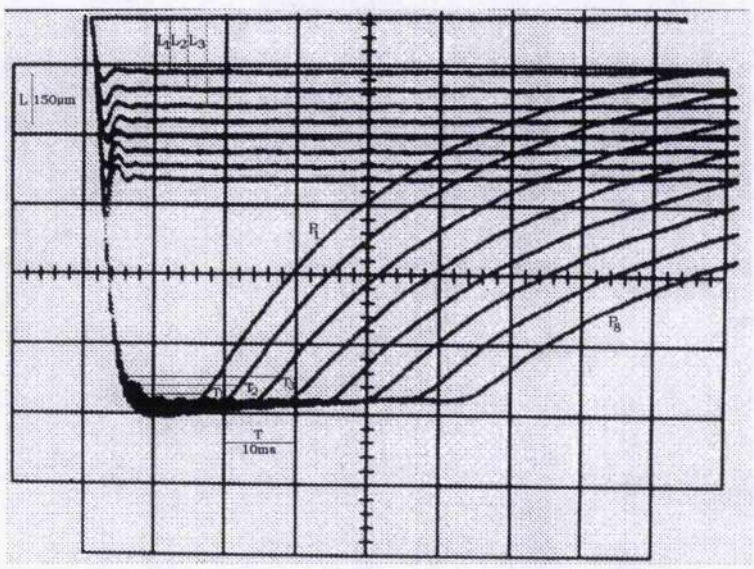
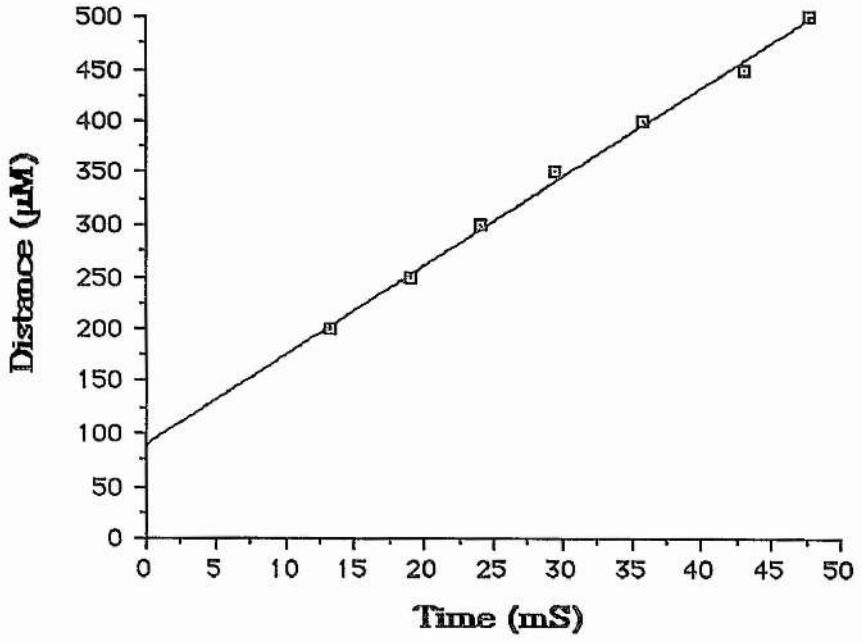


Fig. 2.4: Representative slack test data for a single soleus fibre. V_{max} = 5.03 L_0S^{-1} (Fibre lengths per second). V_{max} is expressed in fibre lengths per second, where fibre length is the end-to-end segment length at which sarcomere length was 2.7-2.8 μm. Correlation coefficient, r=0.997.



EXPERIMENTAL PROTOCOL

The initial experiments were performed at two different temperatures and the concentration of the ionic species and the ionic strength for the solutions used are summarised in Tables 2.1 and 2.2. Although, the amount of added calcium was zero, the calculation for the pCa value of calcium present in the solutions, took into account the small amount of contaminating calcium present in the chemicals used for formulation of the solutions.

Table 2.1: Concentration of the ionic species and the ionic strength at 30 °C, pH 7.0.

Solution	Total [Ca^{2+}] (mM)	pCa	pMg	pMg.ATP	Ionic Strength (mM)
Skinning	0	14.00	3.15	2.25	191.1
Relaxing	0	14.00	3.15	2.25	190.4
Activating	15	4.13	3.08	2.25	199.0

Table 2.2: Concentration of the ionic species and the ionic strength at 25 °C, pH 7.0.

Solution	Total [Ca^{2+}] (mM)	pCa	pMg	pMg.ATP	Ionic Strength (mM)
Skinning	0	14.00	3.12	2.26	191.4
Relaxing	0	14.00	3.12	2.26	190.4
Activating	15	4.14	3.06	2.26	199.3

Firstly, maximal isometric tension (P_o) and V_{max} were determined in a solution of pCa 4.14 for skinned soleus muscle fibres at a temperature of 29-30 °C and in a different population of muscle fibres at a temperature of 24-25 °C. Secondly, P_o and V_{max} were determined at a sarcomere length of 3.05 μm in one population of skinned soleus muscle fibres and at an "optimum" SL of 2.75 μm (Close, 1972; Stephenson &

Williams, 1982) in another population of muscle fibres.

Once the temperature and SL to be used had been decided, as 25 °C and 2.75 μm respectively (throughout the rest of the thesis, the temperature was 25 °C and SL was 2.75 μm for the experiments on single fibres), measurements of P_o and V_{max} were made on fibres dissected from the soleus and TFL muscles isolated from the rat and guinea-pig. Fibres which were taken from muscles on the day of the death of the animal and on which measurements were made are denoted as being "fresh" fibres. Fibres which were isolated from stored bundles of fibres (see below), taken from the freezer on the day of the experiment, are denoted as being "stored" fibres.

STORAGE OF FIBRE BUNDLES

Small bundles of fibres were dissected from soleus or TFL muscles, tied with cotton thread at slightly stretched lengths to glass capillary tubes, placed in 55% v/v glycerol-containing relaxing solution (Moss et al., 1982) and stored at –22 °C for different number of days before use. Single fibres were then taken from these bundles.

The purpose of storing these fibres was to decrease the number of animals used and to sample as many single fibres from one muscle to cater for any biological variability which might exist between fibres of the same muscles from different animals. It would also give a good statistical chance of sampling the different types of fibres present in any one muscle.

HYPERTHYROID AND HYPOTHYROID FIBRES

Once the euthyroid fibres had been characterised with respect to P_o and V_{max}, these same parameters were measured from hyper- and hypothyroid fibres from the soleus and TFL muscles of the rat. In addition, at the end of the measurements, the single fibres were removed onto small diaphragm pieces and identified histochemically as one of three possible groups; FG, FOG or SO (see below). The concentration of the ionic species and the ionic strength of the solutions used have already been presented in Table 2.2.

REMOVAL OF SINGLE FIBRES AFTER MEASUREMENTS

After the dissection of the leg muscles, the diaphragm was quickly dissected. The diaphragm consists of radially arranged fibres running all the way from the tendinous centre to their insertion at the rib cage. The diaphragm was blotted with filter paper, pinned out and allowed to dry by the heat generated by a 60 watt bulb in a table-lamp.

During the course of the day, when the experiment with each single fibre had finished. The fibre was lengthened, using the micro manipulator, to make removal easier. The fibre was then taken out of the relaxing solution and a small rectangular piece of diaphragm, slightly shorter than the length of the fibre and cut parallel to its own fibres, brought up from underneath the fibre, between the two stainless hooks, and the fibre gently eased off from the hooks onto the diaphragm at a 90 degree angle to the fibres of the diaphragm (completed in 15 - 20 sec). The diaphragm with the single fibre was placed in a petri dish on filter paper soaked in 0.9% saline (to keep fibre from dehydration) and placed in the fridge (temperature 0 - 4 °C). This procedure was repeated throughout the day with each single fibre used, at the end of the day,

several rectangular pieces of the diaphragm with individual single fibres were mounted in a sandwich manner onto cryostat chucks in such a way so that the single fibre ends would be cut transversely. Also on one side of the sandwich, a 'blank' (diaphragm without fibre) was mounted to keep the single fibre on the next diaphragm of the sandwich away from the whole muscle section. One whole muscle (dissected at the start of the experiment) section was mounted for support and cross reference on either side and then histochemical staining was performed as described earlier. The rationale of mounting the single fibres together with sections was to provide control values for the density of staining in the individual sections of the fibre that was used for histochemical assay.

The aim of this procedure was to correlate the contractile measurements with the histochemical profile of the fibre.

CALCIUM ACTIVATED TENSION RESPONSES

Three sets of Ca^{2+} activated tension responses were undertaken for fibres from soleus and TFL muscles of hyper- and hypothyroid rats:

a) In solutions of pH 7.0, 0 mM P_i (pH 7.0).

b) In solutions of pH 6.6, 0 mM P_i (pH 6.6).

c) In solutions of 7.5 mM P_i, pH 7.0 (7.5 mM P_i).

pH 7.0, 0 mM P_i (pH 7.0)

For the calcium activated tension responses at pH 7.0, different calcium concentrations ($[Ca^{2+}]$) were obtained by mixing the basic relaxing and activating solutions (Table 2.2) in different proportions (Table 2.3), with this technique a range of Ca^{2+} activating solutions were obtained where $6.75 \geq pCa \geq 4.14$.

Table 2.3: Ratio of relaxing and activating solutions required to achieve the appropriate pCa value at pH 7.0.

Free $[Ca^{2+}]$ (M)	pCa	Relaxing : Activating
1.78×10^{-7}	6.75	9 : 2
3.24×10^{-7}	6.49	5 : 2
8.13×10^{-7}	6.09	1 : 1
1.23×10^{-6}	5.91	2 : 3
2.45×10^{-6}	5.61	1 : 3
7.24×10^{-6}	5.14	1 : 9
7.24×10^{-5}	4.14	0 : 1 (neat)

To characterise the Ca^{2+} sensitivity of tension development in single fibres, the T/pCa relationship was obtained by exposing the fibre sequentially to solutions of increasing free calcium concentrations with the isometric tension being recorded at each $[Ca^{2+}]$. The fibre was relaxed

after each activation in relaxing solution. At the end of the T/pCa relationship, maximal isometric tension ($P_{7.0}$) and maximum velocity of shortening ($V_{max\ 7.0}$) were measured at maximal activation (pCa 4.14, pH 7.0). The concentration of the ionic species and the ionic strength for the activating solutions used at pH 7.0 are summarised in Table 2.4.

Table 2.4: Concentration of the ionic species and the ionic strength of the activating solutions at pH 7.0.

Solution	Total [Ca^{2+}] (mM)	pCa	pMg	pMg.ATP	Ionic Strength (mM)
1	2.72	6.75	3.13	2.26	203.5
2	4.29	6.49	3.13	2.26	203.4
3	7.50	6.09	3.11	2.26	203.3
4	9.00	5.91	3.11	2.26	203.2
5	11.25	5.61	3.10	2.26	203.1
6	13.50	5.14	3.09	2.26	203.1
7	15.00	4.14	3.06	2.27	203.5

pH 6.6, 0mM P_i (pH 6.6)

For the calcium activated tension responses at pH 6.6, different calcium concentrations were obtained, by mixing the basic relaxing and activating solutions (which were at pH 6.6) in different proportions (Table 2.5).

The T/pCa relationships at pH 6.6 were obtained by exposing the fibre sequentially to solutions of increasing free [Ca^{2+}]. At the end of the T/pCa relationship, at pCa 4.10, pH 6.6, isometric tension ($P_{6.6}$) and maximum velocity of shortening ($V_{max\ 6.6}$) were measured. Subsequently the solution was changed to pCa 4.14, pH 7.0 and isometric tension ($P_{7.0}$) and maximum velocity of shortening ($V_{max\ 7.0}$) measured. The concentration of the ionic species and the ionic strength for the

activating solutions used at pH 6.6 are summarised in Table 2.6.

Table 2.5: Ratio of relaxing and activating solutions required to obtain different Ca^{2+} concentrations for T/pCa relationships at pH 6.6.

Free $[Ca^{2+}]$ (M)	pCa	Relaxing : Activating
1.38×10^{-7}	6.86	74 : 1
3.16×10^{-7}	6.50	33 : 1
7.76×10^{-7}	6.11	14 : 1
1.26×10^{-6}	5.90	9 : 1
2.51×10^{-6}	5.60	5 : 1
6.61×10^{-6}	5.18	5 : 2
2.95×10^{-5}	4.53	4 : 3
7.94×10^{-5}	4.10	11 : 10

Table 2.6: Concentration of the ionic species and the ionic strength at pH 6.6 of the different calcium solutions.

Solution	Total $[Ca^{2+}]$ (mM)	pCa	pMg	pMg.ATP	Ionic Strength (mM)
1	0.40	6.86	3.09	2.26	206.7
2	0.88	6.50	3.09	2.26	205.7
3	2.00	6.11	3.09	2.26	203.4
4	3.00	5.90	3.09	2.26	201.3
5	5.00	5.60	3.08	2.26	197.2
6	8.57	5.18	3.08	2.26	199.7
7	12.86	4.53	3.06	2.27	196.0
8	14.29	4.10	3.05	2.27	198.3

7.5 mM P_i, pH 7.0 (7.5 mM P_i)

For the calcium activated tension responses at 7.5 mM P_i, different calcium concentrations were obtained, by mixing the basic relaxing and activating solutions (which were at pH 7.0, 7.5 mM P_i) in different proportions (Table 2.7).

Table 2.7: Ratio of relaxing and activating solutions required to obtain appropriate Ca^{2+} concentrations for T/pCa relationships at 7.5 mM P_i, pH 7.0.

Free $[Ca^{2+}]$ (M)	pCa	Relaxing : Activating
1.78×10^{-7}	6.75	9 : 2
3.24×10^{-7}	6.49	5 : 2
8.13×10^{-7}	6.09	1 : 1
1.23×10^{-6}	5.91	2 : 3
2.45×10^{-6}	5.61	1 : 3
7.24×10^{-6}	5.14	1 : 9
2.63×10^{-5}	4.58	1 : 36
7.24×10^{-5}	4.14	0 : 1 (neat)

T/pCa relationships were obtained at 7.5 mM P_i by exposing the fibre sequentially to solutions of increasing free Ca^{2+} concentrations. At the end of the T/pCa relationship, isometric tension ($P_{7.5mM}$) and maximum velocity of shortening ($V_{max\ 7.5mM}$) were measured. Then the solution was changed to pCa 4.14, 0 mM P_i, pH 7.0 and isometric tension ($P_{7.0}$) and maximum velocity of shortening ($V_{max\ 7.0}$) measured. Concentration of the ionic species and the ionic strength of the solutions used at 7.5 mM P_i are summarised in Table 2.8.

Table 2.8: Concentration of the ionic species and the ionic strength of the activating solutions at 7.5 mM P_i, pH 7.0.

Solution	Total $[Ca^{2+}]$ (mM)	pCa	pMg	pMg.ATP	Ionic Strength (mM)
1	2.73	6.75	3.13	2.26	208.1
2	4.29	6.49	3.12	2.26	207.9
3	7.50	6.09	3.11	2.26	207.5
4	9.00	5.91	3.10	2.26	207.3
5	11.25	5.61	3.10	2.26	207.0
6	13.50	5.14	3.08	2.26	204.7
7	14.60	4.58	3.07	2.26	206.7
8	15.00	4.14	3.06	2.27	207.1

Throughout the experiments for the calcium activated tension responses the sarcomere length was set at 2.75 μm which was the SL used by Stephenson & Williams (1982) for T/pCa relationships for fibres from slow and fast muscles. The operating temperature was 25 °C.

T/pCa RELATIONSHIP CALCULATIONS

The analysis of the results for the T/pCa relationships was undertaken in the following manner:

The effect of $[Ca^{2+}]$ on tension is presented in two ways:

i) The graphical representation of the isometric tension per unit CSA as a function of the pCa (–log $[Ca^{2+}]$). This lets the comparison be made of the absolute contractile strength.

ii) However, the most common method of presenting the effect of $[Ca^{2+}]$ on tension is a graphical representation of relative tension (P_r) as a function of the pCa.

For the relative T/pCa relationships at pH 7.0, P_r was determined in each fibre by expressing isometric tensions per unit CSA at various concentrations of Ca^{2+} as a fraction of the maximum isometric tension per unit CSA ($P_{7.0}$) obtained during maximal activation at pCa 4.14, pH 7.0.

For the relative T/pCa relationships at pH 6.6, relative T/pCa relationships were plotted in two ways:

a) P_r was determined in each fibre by expressing isometric tensions per unit CSA at each [Ca^{2+}] (at pH 6.6) as a fraction of the maximum tension generated in solutions of pH 6.6 ($P_{6.6}$). In the fibres studied at pH 6.6, the maximum tension was always achieved at a free [Ca^{2+}] well below pCa 4.10, and higher calcium led to significantly lower tension. Thus data for these fibres was scaled relative to the actual maximum tension obtained.

b) P_r was determined in each fibre by expressing isometric tensions per unit CSA at each [Ca^{2+}] (at pH 6.6) as a fraction of the maximum tension at pCa 4.14 in solutions of pH 7.0, (i.e., $P_{7.0}$).

The first of these permitted comparisons of the shape of the T/pCa relationships (and hence were used for the construction of Hill plots), and the second was used to compare the effect of pH on tension at each pCa.

For the relative T/pCa relationships at pH 7.0, 7.5mM P_i, relative T/pCa relationships were also plotted in two ways:

a) P_r was determined in each fibre by expressing isometric tensions per unit CSA at each [Ca^{2+}] (at 7.5mM P_i) as a fraction of the maximum tension generated in solutions of pH 7.0, 7.5mM P_i ($P_{7.5mM}$). In the fibres studied at 7.5mM P_i, the maximum tension was always achieved at a free [Ca^{2+}] well below pCa 4.14, and higher calcium led to significantly lower

tension. Thus data for these fibres was scaled relative to the actual maximum tension obtained.

b) P_r was determined in each fibre by expressing isometric tensions per unit CSA at each $[Ca^{2+}]$ (at 7.5mM P_i) as a fraction of the maximum tension at pCa 4.14 in solutions of pH 7.0, (i.e., $P_{7.0}$).

The first of these permitted comparisons of the shape of the T/pCa relationships (and hence were used for the construction of Hill plots) and the second was used to compare the effect of P_i on tension at each pCa.

There were several quantitative characteristics which were indirectly derived from the relative T/pCa relationships:

The indirect characteristics were obtained from average Hill plots of the relative T/pCa relationships. A Hill transformation of the data is a method used to linearize the data for convenient quantitative comparisons.

The T/pCa relationships can be linearized according to the Hill equation:

$$\log P_r / (1- P_r) = n \log [Ca^{2+}] + h.$$

where n and h are constants, and P_r is the relative tension. A straight line is obtained by plotting $\log P_r/(1- P_r)$ against pCa.

Hill plots were constructed by transforming the individual relative T/pCa relationship data to hill plot data and then taking the mean of the results and plotting $\log P_r/(1- P_r)$ as a function of pCa. The calculated quantitative characteristics were:

a) The threshold (equivalent to 3% of maximum tension) for Ca^{2+} activation of tension development (pCa_{TH}) (calculated from the Hill plots as the pCa at which $\log P_r/(1- P_r)$ was equal to −1.5).

b) The pCa_{50} value (calculated from the Hill plots as the pCa at which log $P_r/(1- P_r)$ was equal to 0) which represents the pCa corresponding to 50% of maximum Ca^{2+} activated tension response. Different values indicate a variation of calcium sensitivity associated with the contractile apparatus.

c) The Hill coefficient n (calculated from the slope of the best fit line) represents the number n in the Hill equation which provides the closest fit to the experimental points. The Hill coefficient, n gives an indication of the maximum steepness of the sigmoidal relationship relating P_r to pCa.

The Hill coefficient n, is the assumed number of binding sites acting with maximum co-operativity. Therefore the number of calcium ions involved in the tension regulation is at least equal to n. The largest value should be 4 if all four calcium binding sites of Tn C bind with maximum co-operativity.

MEASUREMENT OF MYOFIBRILLAR ATPase ACTIVITY

The soleus and TFL muscles from both hindlegs were quickly dissected from the treated animals after death. The central portion from one of the soleus muscles was taken for histochemical staining, the remaining portion of the muscle was pooled with the other soleus muscle and weighed. Only one TFL muscle was weighed and used (similar in weight to the combined soleus muscles) for determination of the myofibrillar ATPase activity. The other TFL muscle was used for histochemical staining. Thus, a comparison could be obtained between myofibrillar ATPase activity and general fibre type population differences.

PREPARATION OF MYOFIBRILS

Buffer A: 10 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA
Buffer B: 10 mM Tris-HCl, 100 mM NaCl

The preparation of myofibrils was based on the method of Perry & Grey (1956). All the operations were performed at around 0 °C in an ice-bath. The muscle was cut into small pieces with scissors, placed in a homogenisation vessel and homogenised (Polytron homogeniser) at medium speed in 10-15 vols. of buffer A, for 3 bursts of 30s, the homogenate placed on ice to cool between homogenisations. The homogenate was centrifuged at 6000g for 10 mins, and the pellet washed and re-suspended by a short homogenisation at low speed in 10 - 15 vols of buffer B. The pellet was re-suspended, homogenised and centrifuged as described above a further 3 times. At the end the pellet was homogenised at medium speed in buffer B, for 2 bursts of 30s, the homogenate placed on ice to cool between homogenisations. The myofibrils were contained in this final suspension of the homogenate, and were kept on ice.

PROTEIN ASSAY

The protein content of myofibrils was measured using the microbiuret method based on Itzhaki & Gill (1964). This method is based upon the measurement of the ultraviolet absorption of the complex formed between protein and copper in strongly alkaline copper sulphate solutions.

The method involves taking 2 identical samples of a protein solution. To one is added reagent A, to the other, reagent B. The absorption of both solutions is then read at 310 nm, and that of reagent B + protein (ensures that the contribution to the optical density (OD) of any complex formed outwith the copper/protein complex is negated) subtracted from that of reagent A + protein, giving the final optical density. From this value the protein content can be calculated by comparison with a standard protein calibration curve made with Bovine Serum Albumin (BSA). Readings may be made 5 mins after mixing since the ultraviolet absorption of the copper-protein complex reaches its maximum within this period. The absorption is then constant for at least 2 hours.

Reagent A: solution of 0.21% copper sulphate in 30% sodium hydroxide
Reagent B: solution of 30% sodium hydroxide

1. 2.0 ml dH_2O (Blank)
2. 1.7 ml dH_2O + 0.3 ml BSA (1mg/ml)
3. 1.1 ml dH_2O + 0.9 ml BSA
4. 1.9 ml dH_2O + 0.1 ml myofibril solution

Four sets of the above four test-tubes were prepared, 1 ml of reagent A was added to two sets and 1 ml of reagent B was added to the other two sets. This was so the assay could be done in duplicate.

All the mixtures were mixed on a vortex and the optical density measured after 10 mins in a spectrophotometer (CECIL) at 310 nm in 1 cm spectrophotometer cells. The absorption of the sample or standard against the appropriate blank was read and that of reagent B + protein subtracted from that of reagent A + protein, giving the optical density. From this value the protein concentration was calculated by reference to a standard protein calibration curve constructed with BSA in the range of 0.025 mg/ml to 0.5 mg/ml. Two standard BSA tubes were run with each assay to act as controls.

MYOFIBRILLAR ATPase ASSAY

The ATPase activity was measured by a modification of the method of White (1982) and carried out at room temperature. All measurements of ATPase activity were performed on the same day that the myofibrils were prepared.

Solutions used:

1. Myofibrils (5mg/ml) in 100 mM NaCl, 10 mM Tris-HCl, pH 7.2
2. Ca^{2+} buffer: 62.5 mM Tris-HCl, 3.8 mM $MgCl_2$, 0.2 mM $CaCl_2$, pH 7.0 at 25 °C
3. EGTA buffer: 62.5 mM Tris-HCl, 3.8 mM $MgCl_2$, 0.2 mM EGTA, pH 7.0 at 25 °C
4. 13.3% SDS, 0.12 M EDTA, pH 7.0
5. 20 mM Disodium ATP at pH 7.0

To 0.55 ml of Ca^{2+} buffer or EGTA buffer, in a test-tube, was added 0.1ml of the myofibril solution, this was mixed in a vortex and the reaction started by the addition of 0.1 ml ATP, the reaction was stopped in different test-tubes after time intervals of 1 min up to 5 mins by the addition of 0.25 ml SDS (sodium dodecyl sulphate). At the beginning and during the reaction the test-tubes were periodically (every 15 secs) vortexed to stop any local build-up of metabolites, such as ADP and P_i, which may shift the equilibrium of the reaction. Once the reaction was stopped the amount of P_i was determined by the phosphate assay (see below). Appropriate blanks were also taken, i.e. where the SDS was added before the addition of ATP, this allows for background contamination and any spontaneous hydrolysis of ATP. The assays were performed in duplicate.

PHOSPHATE ASSAY

Solution used:

1. 0.5% (w/v) ferrous sulphate, 0.5% (w/v) ammonium molybdate. 0.5 M H_2SO_4, made fresh daily from a solution of 10% ammonium molybdate, 10 M H_2SO_4 (stable up to 6 months at room temperature), solid ferrous sulphate, and distilled H_2O.

The phosphate assay was based on the method of Rockstein & Heron (1951). To the 1 ml sample containing P_i (from the myofibrillar ATPase incubation) was added 2 ml of solution 1. This initiated the colour development which was complete after 15 mins and stable for at least 60 mins. The absorbance of the samples was read at 550 nm after 20 mins.

The amount of P_i liberated was calculated from a curve constructed between 25 nmoles to 1000 nmoles of phosphate, by using standard phosphate solutions in the place of the myofibril solution, and distilled H_2O in place of the ATP solution. The results for the myofibrillar ATPase assays were plotted as μmoles of P_i released/mg protein against time. The rate of P_i release was calculated from the slope of the initial straight line (drawn between 0 and 2 minutes) portion of the rate curve. The results were expressed in μmoles of P_i released/mg protein/min.

CALCIUM SENSITIVITY

Calcium sensitivity shows how much activity is produced by the calcium independent component of myofibrillar ATPase activity and shows how active troponin C is in inhibiting the activity since calcium relieves the inhibition of troponin C for muscle contraction.

The calcium sensitivity was calculated as follows:-

$$\text{Calcium sensitivity (\%)} = \left\{ 1 - \frac{\text{EGTA } Mg^{2+} \text{ ATP}_{ase}}{Ca^{2+} Mg^{2+} \text{ ATP}_{ase}} \right\} \times 100$$

HISTOCHEMICAL CALCULATIONS

The slides were examined under a microscope and the fibres from a whole muscle were traced out onto paper using the arm of a microscope at a 20-fold magnification for the soleus and 10-fold for the TFL. Three different sections of the muscle were examined and the fibres were classified as slow oxidative (SO), fast oxidative glycolytic (FOG) or fast glycolytic (FG) on the basis of staining reactions for SDH and myosin ATPase.

Due to the homogeneous distribution of fibre types within the muscles and the fact that the number of fibres in the slow and fast hyper- and hypothyroid muscles have been shown to remain constant (Nicol & Johnston, 1981), Three sections, selected from different areas, of the muscle were sufficient to obtain accurate fibre type populations.

RESULTS & DISCUSSION

CONTRACTILE PROPERTIES OF EUTHYROID SOLEUS AND TFL FIBRES

The format of the results presented in this thesis is (unless otherwise indicated) as follows:

1. Results are reported as the mean ± standard error of the mean (SEM).
2. N is the number of animals used.
3. The number of observations are indicated in parenthesis.
4. Differences between groups are determined by the unpaired two tailed student's t-test. Significance is accepted at $P < 0.05$. Two different levels of significance were calculated between groups, either $P < 0.05$, denoted by * or $P < 0.005$, denoted by +.

RAT SOLEUS MUSCLE FIBRES AT TWO DIFFERENT TEMPERATURES

The results (Table 3.1) show that there was no significant difference in either the isometric tension (mg) or the maximum isometric tension produced per unit cross-sectional area (P_o) or in the maximum velocity of shortening (V_{max}) when the operating temperature was reduced from 30 °C to 25 °C.

It can also be seen that there was no difference in the mass of the rats or the diameter of the single muscle fibres used for the two sets of experiments.

Table 3.1: Measurements of euthyroid rat soleus fibres at two different temperatures. The sarcomere length (SL) of the fibres was set at 3.05 μm.

	Temperature	
	30 °C	25 °C
Rat weight (g)	376.98 ± 10.33 (N = 5)	397.28 ± 18.29 (N = 14)
Fibre diameter (μm)	58.26 ± 2.10 (42)	57.04 ± 1.32 (107)
CSA x 10^{-5} (cm^2)	2.81 ± 0.25 (42)	2.70 ± 0.14 (107)
Tension (mg)	34.82 ± 2.32 (42)	32.06 ± 1.14 (107)
P_o (kN/m^2)	133.05 ± 8.33 (42)	129.07 ± 4.77 (107)
V_{max} (L_oS^{-1})	5.07 ± 0.70 (17)	5.64 ± 0.31 (43)

The temperature dependence of a rate process or a measured quantity is expressed as its temperature coefficient (Q_{10}) where:

$$Q_{10} = \left(\frac{RM_2}{RM_1} \right)^{\left(\frac{10}{(T_2 - T_1)} \right)}$$

in which RM_2 and RM_1 are rate processes or the quantities measured, at temperatures T_2 and T_1 respectively, and $T_2 > T_1$. $Q_{10} > 1.0$ indicates a positive thermal dependence; $Q_{10} = 1.0$ indicates thermal independence and $Q_{10} < 1.0$ indicates declining function with increasing temperature.

The Q_{10} for P_o was 1.06 indicating thermal independence. This observation is consistent with results obtained in whole muscle preparations (Buller et al., 1968; Close & Hoh, 1968; Close, 1972; Hoh, 1974; Issacson et al., 1970; Ranatunga, 1977, 1980). Furthermore, a study (Stephenson & Williams, 1981) on the same kind of preparation as the present one, and is therefore more comparable, found that in skinned muscle fibres isolated from rat fast (Extensor digitorum longus – EDL) and slow (soleus) muscles, P_o only increased slightly (non significant) over the temperature range 25 - 35 °C. This result is in total agreement

with the present study which has found a Q_{10} value of 1.06 (a slight non significant increase for the soleus) over the temperature range 25 – 30°C.

The temperature sensitivity of tension seems to be a property of the cross bridges rather than the activation system. According to the sliding filament-attached cross bridge model of contraction, tension depends on the number of attached cross bridges and the average tension per cross bridge (Huxley, 1957). Stiffness in skeletal muscle is less sensitive to changes in temperature than is tension (Goldman et al., 1987) and since stiffness of a muscle during contraction has been taken as an indicator of the number of attached cross bridges (Ford et al, 1981). This suggests that the tension generating capacity of a cross bridge increases with temperature. According to this argument, the primary explanation for an increased tension with increased temperature is augmented tension per cross bridge rather than an increase in the number of cross bridges.

The Q_{10} for V_{max} was 0.81 (non significant) which indicates a decrease with an increase in temperature. This observation is in conflict with results obtained by previous investigators (Faulkner, 1980; Ranatunga, 1982). They found that V_{max} decreased with cooling over the temperature range 35 - 25 °C in the soleus of the rat and the EDL of mice respectively. However, both of these studies used multi–fibre preparations and are therefore more than likely to contain a heterogeneous fibre population with respect to intrinsic shortening velocity. Thus it is possible that temperature will affect differently the various fibres present in these types of preparations. Despite this, we feel that this is not enough to explain the result obtained because studies on single fibres in frog muscle (Cecchi et al., 1978) and fish muscle (Johnston & Sidell, 1984) have shown that V_{max} has a positive thermal dependence. Therefore, the present result could be a consequence of i)

the study is limited to only a 5 °C change and the results are not significant which can only be taken as being suggestive or ii) the method employed. The former of these explanations is more likely than the latter.

RAT SOLEUS MUSCLE FIBRES AT TWO DIFFERENT SARCOMERE LENGTHS

The results (Table 3.2) show that there was a significant increase in tension and in P_o of 17% when the sarcomere length was reduced from 3.05 to 2.75 μm. But there was no significant difference in V_{max} at the two different sarcomere lengths.

Table 3.2: Measurements of euthyroid rat soleus fibres at two different sarcomere lengths. The operating temperature was 25°C.

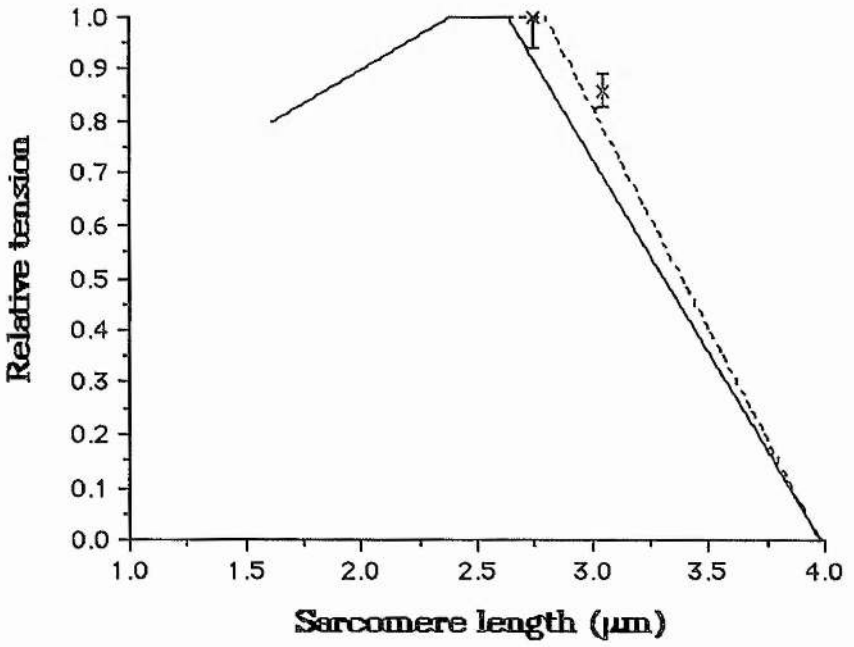
	Sarcomere length (SL)	
	2.75 μm	3.05 μm
Rat weight (g)	384.57 ± 24.52 (N = 10)	397.28 ± 18.29 (N = 14)
Fibre diameter (μm)	55.89 ± 1.55 (42)	57.04 ± 1.32 (107)
CSA x 10^{-5} (cm^2)	2.53 ± 0.15 (42)	2.70 ± 0.14 (107)
Tension (mg)	37.37 ± 2.67 (42) *	32.06 ± 1.14 (107)
P_o (kN/m^2)	150.12 ± 8.49 (42) *	129.07 ± 4.77 (107)
V_{max} (L_oS^{-1})	5.61 ± 0.54 (32)	5.64 ± 0.31 (43)

* $P < 0.05$, 2.75 μm vs. 3.05 μm

The observation that P_o of the maximum calcium activated skinned fibres of the soleus increased with a decrease of SL (Fig. 3.1) from 3.05μm (near the top of the descending limb) to 2.75μm (plateau region) is consistent with the length-tension diagrams obtained in intact fibre bundles (ter Keurs et al., 1981) and skinned muscle fibres of the rat (Stephenson & Williams, 1982). In general terms the result agrees with the theoretical length–tension relation (Fig. 3.1) which can be constructed using the rationale of Gordon et al. (1966a), assuming the

bare zone in the middle of the myosin filament is 0.25 μm and that the total lengths of the thick and thin filaments are 1.6 and 2.38 μm respectively (Page & Huxley, 1963; Close, 1972; Luff et al., 1981; ter Keurs et al., 1981). The small deviation between the experimental and predicted result is probably due to variations in the sarcomere length within the fully activated skinned fibre preparation (Julian & Moss, 1980).

Fig. 3.1: Graphical representation of the isometric tension– sarcomere length relation in the rat. The solid line is the predicted tension–length curve. The dotted line, are results from Stephenson & Williams (1982). x represents the results from the present study with error bars representing ± SEM



The result that V_{max} of the skinned fibres of the soleus remained unchanged when the SL was reduced is in agreement with the result on intact single frog muscle fibres (Edman, 1979). This result can be understood from the limited range of action of the cross bridge. Each cross bridge has a relation between the tension it exerts and its length. Its length depends on the position in which it formed, and on any relative movement of the thick and thin filaments that has taken place

since then. When it is formed a cross bridge presumably exerts tension in the forward direction (a pull), but, if shortening occurs while it remains attached, the tension will fall. If this process continues, the tension will fall to zero and then reverse. The bridge will then be "pushing". Thus, particularly during rapid shortening the filaments experience tensions in both directions from the cross bridges. At V_{max}, where there is no external tension opposing shortening, the forward and backward tensions balance. Therefore, V_{max} should be independent of the number of attached cross bridges, and, consequently independent of filament overlap. When the muscle is shortening at V_{max}, cross bridges can remain attached for less time than during isometric contraction. Since presumably a finite time is required for the reattachment of a broken cross bridge, the number attached should be fewer during shortening. In agreement with this expectation, it is found that the stiffness of rapidly shortening muscle is less than that of isometrically contracting muscle (Julian & Sollins, 1975), and that the relative intensity of the 1 : 1 and 1 : 0 reflections is changed in a way that is compatible with there being fewer cross-bridges attached (Huxley, 1979).

The present results (e.g. the effect on P_o when temperature or SL was changed) agree favourably with previous studies where measurements were made on the same preparation when temperature or SL was changed. This suggests that properties that apply in one preparation can also be seen in fibres from two different populations even though there is variability between fibres from muscles of different animals and variability within the same muscle between fibres. In the present study this variability has probably been overcome because of a number of factors combining together, i) the weight and the age of the animals used was similar ii) the large number of observations made and

iii) the result that the diameter of the fibres between the changes in temperature or SL remained unchanged, suggests that the fibres sampled were of a similar type. This is likely to be the SO type due to the histochemical profile of the rat soleus, which is 84% SO and 16% FOG (Ariano et al., 1973).

It has been shown that both slow and fast muscle fibres are unaffected by a temperature change between 35 – 25 °C (Stephenson & Williams, 1981) and there is no apparent difference between the SL-tension relation in slow and fast twitch skinned muscles (Stephenson & Williams, 1982). Therefore, Once the optimum conditions were established as 25 °C for the temperature and 2.75μm for SL. Measurements were made for the soleus and TFL muscle fibres from the two rodents. Thereafter, all subsequent experiments on single fibres were performed at optimum conditions.

For the muscles from the rat, the tension was 56% higher in TFL muscle fibres than in soleus muscle fibres (Table 3.3). However, when expressed per unit CSA, P_o of the TFL muscle fibres was 16% lower than the soleus muscle fibres. The diameter was 35% higher for the TFL muscle fibres as opposed to the soleus muscle fibres and the TFL fibres had a 2.20-fold higher V_{max} value than the soleus fibres.

Table 3.3: Measurements from euthyroid rat soleus and TFL muscle fibres.

	Soleus	TFL
Rat weight (g)	384.57 ± 24.52 (N = 10)	377.30 ± 17.25 (N = 5)
Fibre diameter (μm)	55.89 ± 1.55 (42) +	75.70 ± 2.27 (25)
CSA x 10^{-5} (cm^2)	2.53 ± 0.15 (42) +	4.60 ± 0.28 (25)
Tension (mg)	37.37 ± 2.67 (42) *	58.16 ± 4.07 (25)
P_o (kN/m^2)	150.12 ± 8.49 (42) *	125.70 ± 6.16 (25)
V_{max} (L_oS^{-1})	5.61 ± 0.54 (32) +	12.35 ± 0.95 (16)

* $P < 0.05$, + $P < 0.005$, soleus vs. TFL

For the muscles from the guinea-pig (GP), the tension was significantly higher (45%) in TFL muscle fibres than soleus muscle fibres (Table 3.4). When expressed per unit CSA the tension was slightly higher (12%) in TFL than in soleus muscle fibres. The diameter and hence the CSA of the TFL muscle fibres was higher than the soleus muscle fibres by 16% and 31% respectively. The V_{max} of the TFL fibres was 2.5-fold higher than the soleus fibres. Finally, the weight of the animals used was very similar.

Table 3.4: Measurements of euthyroid guinea-pig soleus and TFL muscle fibres

	Soleus	TFL
Guinea-pig weight (g)	450.83 ± 61.78 (N = 6)	452.52 ± 73.76 (N = 5)
Fibre diameter (μm)	49.63 ± 2.22 (27) *	57.37 ± 2.96 (14)
CSA x 10^{-5} (cm^2)	2.04 ± 0.18 (27)	2.67 ± 0.30 (14)
Tension (mg)	27.74 ± 2.60 (27) *	40.29 ± 4.37 (14)
P_o (kN/m^2)	135.46 ± 6.52 (27)	152.12 ± 11.39 (14)
V_{max} (L_oS^{-1})	3.92 ± 0.35 (18) +	9.93 ± 1.14 (7)

* $P < 0.05$, + $P < 0.005$, soleus vs. TFL

The fibres sampled from the soleus and TFL muscles from both rodents would probably have been, of the SO type from the former and FG type from the latter. This is primarily due to the histochemical profile of the muscles. For in the GP, the soleus is totally composed of SO fibres whereas in the rat 84% are of the SO type and 16% are of the FOG type. On the other hand, the TFL muscles of the GP have 81% FG and 19% FOG fibres, and the TFL muscles of the rat have 94% FG and 6% FOG fibres (Ariano et al., 1973). Therefore, the majority fibre type is SO in the soleus and FG in the TFL muscles. Secondly, the results from hyper- and hypothyroid fibres (present study), show that where single fibres have been identified histochemically, the fibres isolated from the rat soleus and TFL muscles have been identified as SO type and FG type respectively.

The results show that skinned rat and guinea-pig soleus and TFL muscle fibres (i.e., SO and FG respectively) generated tensions in a narrow range of 125 – 150 kN/m^2. The values obtained are quantitatively comparable to those observed in earlier studies on similar types of preparations (Sweeney et al., 1986, 1988; Reiser et al., 1987b; Metzger & Moss, 1987; Mounier et al., 1989). However, one study (Stephenson &

Williams, 1982) has reported tension values twice as large with similar types of preparations, which could be due to the mechanical skinning or the composition of the solutions employed in that study.

The maximal tension results obtained for the two types of muscle fibres from the GP are in general agreement with two previous studies (Gulati, 1976; Takagi & Endo, 1977), whereas they contradict the results obtained by Powell et al. (1984), who showed a larger tension per unit CSA for the fast rather than the slow muscles. For the rat, the results agree with those obtained by Gardetto et al. (1989) on single muscle fibres, but are in contradiction to the results obtained by many previous investigators.

First of all, on the whole muscle level, rat fast muscle has been shown to generate larger tension than slow muscle (Close, 1972; Powell et al., 1984). Although, a recent study by Ranatunga & Thomas (1990) has shown no apparent difference in the tension generated by the two types of muscles. A similar scenario, exists at the single fibre level with some investigators (Stephenson & Williams, 1982; Mounier et al., 1989) observing about 40% larger tensions in fast than slow muscle fibres, whilst others (Takagi & Endo, 1977; Reiser et al., 1987a, b; Metzger & Moss, 1987; Sweeney et al., 1988) have observed no significant difference.

Although, differences exist in the literature with respect to the tension generating capacity of fibres from slow and fast mammalian muscles, it is generally agreed that there is no marked difference in the intrinsic strength of the cross bridge from different fibre types (Close, 1972; Ranatunga, 1984; Sweeney et al., 1986, 1988; Lucas et al., 1987; Greaser et al., 1988; Chamberlain & Lewis, 1989) or any large difference in the contractile material per unit mass (Close, 1972). Therefore, any apparent differences obtained are due to extrinsic factors that influence activation.

However, the difference observed between the soleus and rat muscle fibres in the present study is unlikely to be explained on the basis of extrinsic factors, since all the solutions and procedures were standardised. Therefore, the present results are likely to reflect a difference in either the number of cross bridges and/or tension of the individual cross bridge.

As far as the V_{max} values were concerned for the two different rodents, results obtained were consistent with earlier studies on single muscle fibres (Reiser et al., 1985a, b; Sweeney et al., 1986, 1988) which found fibres from fast muscles to have a 2–3 fold higher V_{max} than fibres from slow muscles. The higher V_{max} of the fast muscle fibres is due to the higher ATPase activity of the fast myosin isoenzyme (Barany, 1967).

It was also found that the V_{max} values of the soleus or TFL fibres of the rat were higher than that of the GP respectively. This is consistent with the results obtained by Asmussen & Marechal (1989) and is to be expected on the basis of the weights of the rodents, since speed of contraction is directly related to size of the animal (Close, 1972). So since, GPs are generally heavier than rats the V_{max} of the same muscles will be lower in the GPs than the rats and this is reflected in the present study at the single fibre level.

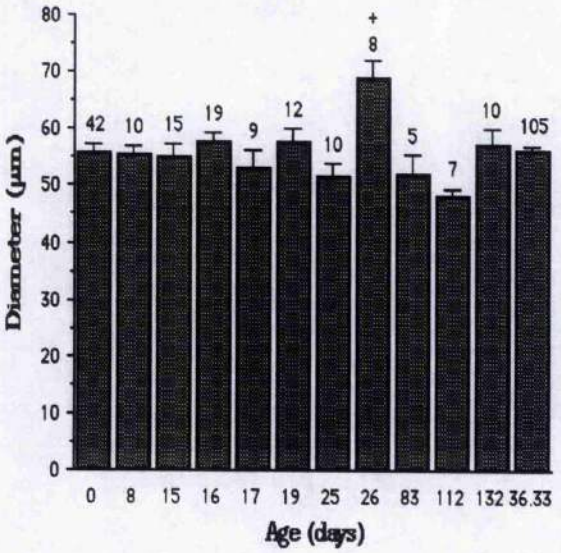
In figure 3.2, the results of stored fibres have been compared with fresh fibres (0 days). The number of observations are indicated above each column, and the symbols above the column represent the levels of significance (* $P < 0.05$ or + $P < 0.005$) between fresh and stored values. The results are shown with + SEM bars. The column on the extreme right handside represents the mean + SEM of all the observations made on stored muscle fibres. The – SEM (hidden within the columns) is not shown but is equal and opposite to + SEM. The extreme right handside column value, which is an average of the results of all the stored muscle fibres irrespective of the time stored, is indicative of any trend to be seen and it will have a decreased ± SEM due to a larger number of observations.

Soleus muscle fibres

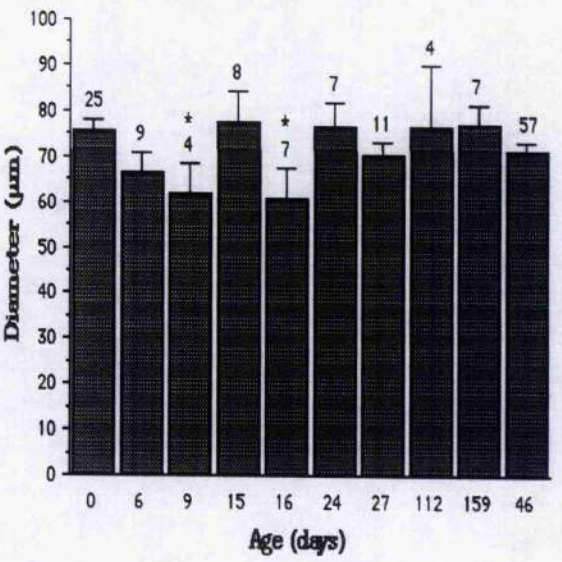
There was no effect on the diameter (Fig. 3.2a) of soleus muscle fibres in the first 3 weeks of storage. Although, a significant increase of 23% was seen after 26 days, but this was not sustained at longer storage periods, where the diameter of the fibres was essentially unchanged.

Fig. 3.2: Effect of storage on the diameter, maximum isometric tension per unit cross-sectional area (P_0), and V_{max} of single skinned soleus a) to c) respectively and TFL d) to f) respectively muscle fibres of the euthyroid rat. Average weight of the animals used for the soleus muscles was 366.22 ± 17.60 (9) and for the TFL muscles was 369.22 ± 24.48 (6).

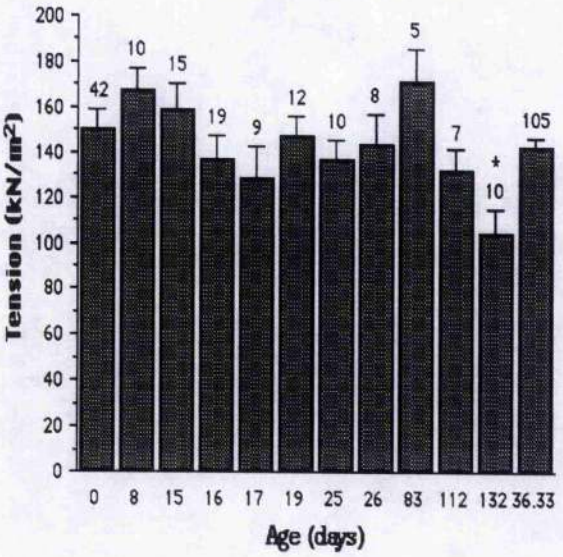
a) Soleus fibres



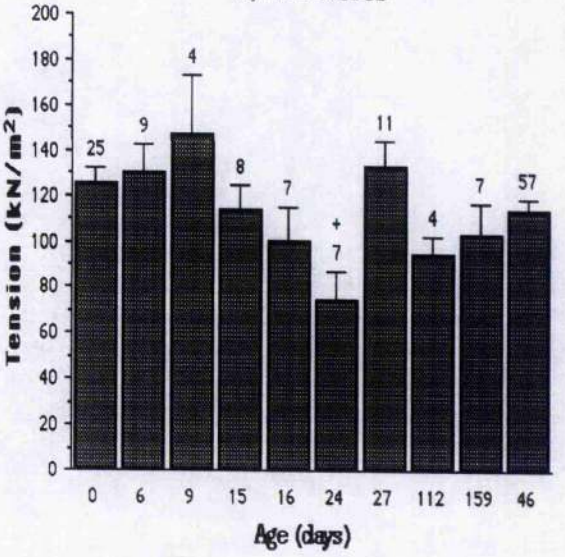
d) TFL fibres



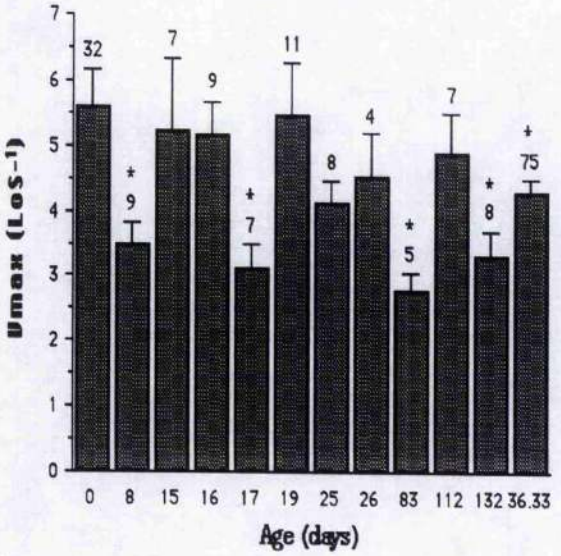
b) Soleus fibres



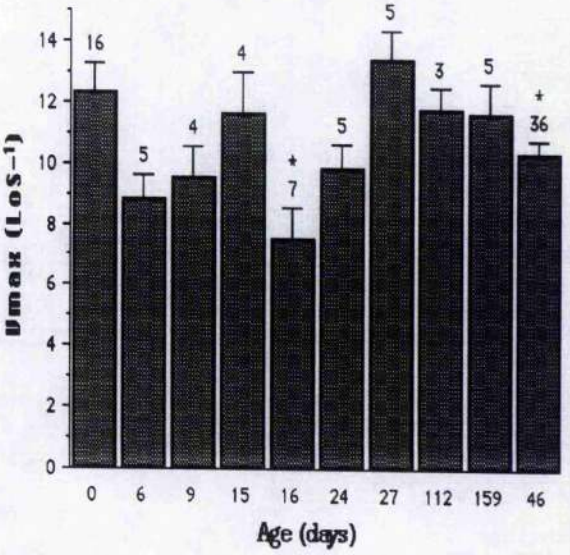
e) TFL fibres



c) Soleus fibres



f) TFL fibres



There was also no effect of storage on the isometric tension per unit cross sectional area (Fig. 3.2b) of the fibres for a period of 3 months, with only a significant decrease of 30% seen after 4 months of storage. The decrease seen in the tension after 4 months is probably due to the effects of glycerol on the contractile proteins and is not due to any effect of storage on the diameter.

On the other hand, all the V_{max} (Fig. 3.2c) values (not all were significant) of stored muscle fibres were lower than the fresh fibre values. But there was no linear or other apparent relationship to describe the lower values observed. A significant decrease of 38% (8 days) and 44% (17 days) was seen when the fibres had been stored for short periods (weeks), and a significant decrease of 51% (83 days) and 41% (132 days) was also seen when the fibres had been stored for longer periods (months). The value in the extreme right handside column for V_{max}, which is an average of the cumulative values of all the stored muscle fibres, was also significantly lower by 24% with respect to fresh fibres, whereas the average value for the diameter and tension of stored fibres remained unchanged with respect to fresh fibres.

TFL muscle fibres

A significant decrease of 18% (9 days) and 20% (16 days) was seen during the shorter period of storage, in the diameter of TFL muscle fibres (Fig. 3.2d). However, the diameter of all the other fibres whether stored for short or long periods remained unchanged. The decrease in diameter is probably within the range of sampling, because if anything glycerol would be expected to increase fibre diameter due to the swelling which might occur.

There was also on the whole no change in the tension of the fibres (Fig. 3.2e) except for a significant decrease of 40% after 24 days of storage. The value in the average column on the extreme right handside, for both the diameter and tension was essentially unchanged with respect to fresh fibres. On the other hand, all but one (27 days) of the V_{max} values (Fig. 3.2f) of stored muscle fibres was lower than the fresh fibre values. During the shorter period of storage, a decrease of 28% (6 days), 23% (9 days), 39% (16 days) and 21% (24 days) was seen in the values of V_{max} (only the value at 16 days was significantly lower), whilst in the longer periods of storage the values of V_{max} remained unchanged. The average value of V_{max} on the extreme right handside was significantly lower by 14% with respect to the fresh fibres.

The results (not shown) for the GP (soleus and TFL fibres) were similar to those obtained for the rat. Generally, in the GP, the diameter was unchanged but V_{max} was significantly reduced. However, isometric tension was reduced in the GP whilst it remained unchanged in the rat.

The glycerol treatment did not affect the diameter in any manner, thus it probably reduced tension either by reducing the number of active cross bridges and/or reducing the intrinsic strength of each cross bridge. V_{max} was probably affected due to the partial inactivation of the myofibrillar ATPase activity by denaturation of the proteins. In this respect, Johnston et al. (1980a) reported that the absolute activities obtained with myosin prepared from frozen muscles were lower than those from fresh muscles.

In view of these results it was thought better to undertake experiments on fresh fibres because of the definite effect of storage on V_{max} in the rat and GP. In addition, it has been shown (Nakayama et al., 1983) that glycerinated fibres lose Ca^{2+} dependent regulation of muscle

contraction and in view of the experiments to be conducted on the tension-pCa relationships it was better to use fresh fibres.

MAXIMAL ISOMETRIC TENSION AND V_{max} OF HYPO- AND HYPERTHYROID RATS

HISTOCHEMICALLY IDENTIFIED SOLEUS AND TFL MUSCLE FIBRES

For both the soleus and TFL fibre measurements, the weight of the hyperthyroid animals was slightly (9%) but significantly ($P < 0.05$) higher than the weight of the hypothyroid animals (Table 4.1), although only mild forms of treatment were administered.

Table 4.1: Measurements of single soleus and TFL fibres from rats of different thyroid status.

	Hyperthyroid	Hypothyroid
	SOLEUS	
Rat weight (g)	404.14 ± 13.36 (N = 7) *	370.00 ± 6.95 (N = 8)
Core temperature (°C)	37.86 ± 0.18 (7) +	36.31 ± 0.13 (8)
Fibre diameter (µm)	58.07 ± 0.98 (67)	56.51 ± 0.92 (83)
CSA x 10^{-5} (cm^2)	2.70 ± 0.09 (67)	2.56 ± 0.08 (83)
Tension (mg)	34.27 ± 1.67 (67) +	43.81 ± 2.10 (83)
P_o (kN/m^2)	127.12 ± 5.87 (67) +	169.02 ± 6.62 (83)
V_{max} (L_oS^{-1})	6.58 ± 0.32 (51) *	5.52 ± 0.27 (55)
	TFL	
Rat weight (g)	413.83 ± 7.86 (N = 6) *	369.67 ± 17.25 (N = 6)
Core temperature (°C)	37.18 ± 0.34 (6) *	36.25 ± 0.17 (6)
Fibre diameter (µm)	69.97 ± 2.14 (40)	69.75 ± 1.92 (37)
CSA x 10^{-5} (cm^2)	3.99 ± 0.27 (40)	4.03 ± 0.26 (37)
Tension (mg)	47.08 ± 2.87 (40) *	55.25 ± 2.41 (37)
P_o (kN/m^2)	121.30 ± 6.80 (40) *	142.73 ± 6.38 (37)
V_{max} (L_oS^{-1})	13.81 ± 0.50 (34) *	11.18 ± 0.65 (7)

* $P < 0.05$, + $P < 0.005$, hyper- vs. hypothyroid

For both sets of experiments, the core temperature was significantly lower in hypothyroid than in the hyperthyroid animals.

The slight, but significant, higher weights observed in the hyperthyroid animals compared to the hypothyroid animals is probably due to the range of weights (230 – 270 g) of the animals taken before the treatment was started. Since the protocol used to induce hyper- or hypothyroidism has been found to show no significant difference between the weights of hyper- and hypothyroid animals (Nicol & Johnston, 1981). In essence, the treated animals remained in a net anabolic state throughout the six week period of treatment, since treated animals had weights which were not significantly different from euthyroid animals. The thyroid hormone levels were not measured, as they had been shown, previously, (Nicol & Bruce, 1981; Nicol & Johnston, 1981) using the same method of treatment, to increase to 2.979 ± 0.443 ng/ml in thyroid treated animals and decrease to 0.56 ± 0.004 ng/ml in thyroid deficient animals. However, as a precaution the core temperature of the treated animals was taken at death. This is an indirect measure of the basal metabolic rate, which is known to increase in hyperthyroid and decrease in hypothyroid animals (Winder et al., 1975; Janssen et al., 1978). The basal metabolic rate can be increased by upto 100% when the levels of circulating thyroid hormones are higher than normal. Conversely it can be decreased as much as 50-60% in severe hypothyroidism. Associated with changes in the basal metabolic rate, are changes in oxygen consumption and the production of heat (Baldwin et al., 1978; Janssen et al., 1978).

It must be pointed out that the hyperthyroid animals would be expected to have an unchanged core temperature with respect to euthyroid animals and it would be the hypothyroid animals which would have a slightly lower core temperature with respect to eu- and

hyperthyroid animals. Therefore, the lower core temperature of hypo- than of hyperthyroid animals in the present study confirms the thyroid status of the animals.

Histochemically, none of the fibres sampled from the soleus muscle of treated animals were identified as being of the FOG type, thus all the fibres sampled were of the SO type (Plate 4.1). Similarly, all the fibres sampled from TFL muscles of treated animals were of the FG type with none being identified as the FOG type.

To understand this, it must be borne in mind that firstly the number of fibres used from any individual muscle was between 5 to 12 depending on how easy it was to dissect the single fibres. Therefore, from a muscle containing about 2500-4000 muscle fibres (Nicol & Bruce, 1981) only a tiny number of fibres was being sampled, this problem was unavoidable since the muscle fibres could not be stored for any length of time. Secondly the size of the SO and FG fibres was larger (Nicol & Bruce, 1981; Sickles et al., 1987), therefore it was much easier to dissect these, although an effort was made to dissect smaller sized fibres it was very difficult to distinguish between the sizes until actual measurements were made and as can be seen (Nicol & Bruce, 1981; Nicol & Johnston, 1981; present study) there is not a large difference in the sizes of the different types of fibres in a muscle. Thirdly the histochemical profile of the muscles used was such that the FOG fibres were always present as a low minority (present study).

Alternatively, it could be that the FOG type fibres cannot be identified using this technique, but this is highly unlikely, since they were present when small bundles (5-15 fibres) of fibres were taken and analysed histochemically.

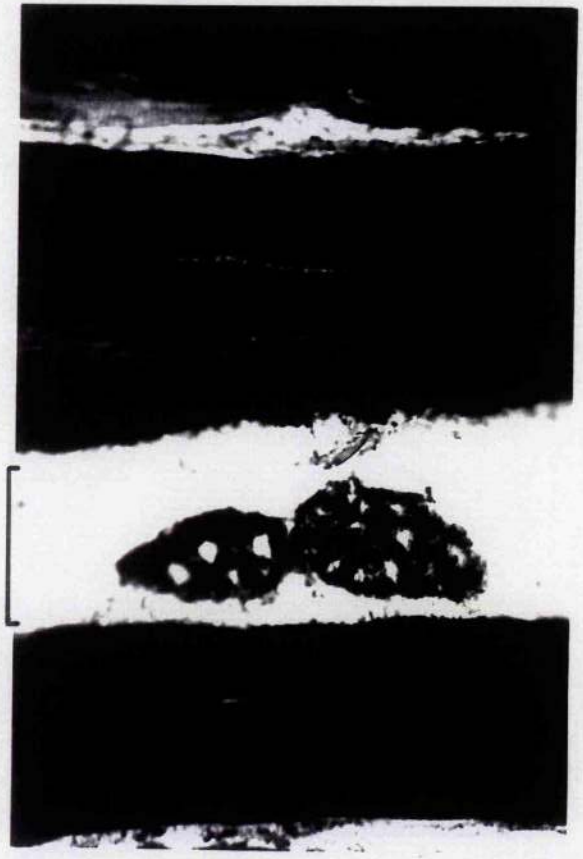
Plate 4.1: Photographs of single muscle fibres from soleus muscle. Scale bar represents 50 μm.

a) SDH stain

b) Acid pre-incubated ATPase stain

c) ATPase stain (neutral fixation)

The fibres shown in the photographs were identified as being of the SO (Slow Oxidative) type. They stain moderately, intensely and lightly in the SDH, acid pre-incubated and neutrally fixed stains respectively.



There was no significant difference in the diameter of the fibres sampled between the two thyroid states in either the fibres from the soleus or TFL muscles (Table 4.1). Firstly, this suggests the same type of fibres were sampled in the different thyroid states, concurring with the histochemical results obtained. Otherwise, a decreased diameter would have been observed for hyper- soleus vs. hypothyroid soleus fibres and a decreased diameter would have been observed for hyper- or hypothyroid TFL vs. euthyroid TFL fibres. Since it is known that different muscle fibre types have different sizes, e.g., in soleus, SO fibres are larger than FOG fibres (Nicol & Bruce, 1981; present study) and FG fibres are larger than FOG fibres in TFL (Sickles et al., 1987; present study). Fibre inter conversion occurs from the large sized SO fibres to the small sized FOG fibres in the hyper- soleus muscles and vice versa in the hypothyroid soleus (Nicol & Bruce, 1981; Nicol & Johnston 1981), Whereas in the TFL, The large sized FG fibres inter convert to small sized FOG fibres in both treated states (present study). Secondly, it suggests that mild dysthyreosis does not affect the diameter of the same type of fibres in any significant manner, showing that no large scale structural changes occur in the fibre.

In view of the above results, for the purpose of this study all fibres taken from the soleus muscles will be treated as being of the SO type whereas fibres taken from the TFL muscles will be treated as being of the FG type, unless there is evidence to suggest otherwise e.g., the diameter of the fibres sampled. Therefore any change that is seen is due to the effect of dysthyreosis on the SO or FG type fibre, as opposed to an effect being due to fibre conversion, which is known to occur as shown by whole muscle histochemistry (Nicol & Johnston, 1981; Nicol & Bruce, 1981; Nicol & Maybee, 1982; present study).

In both types of muscles, fibres from hyperthyroid animals were found to generate less maximum isometric tension than fibres from hypothyroid animals (Table 4.1). These results agree with maximum isometric twitch tensions obtained by authors working on whole muscles (Gold et. al., 1970; Fitts et. al., 1980, 1984; Everts, 1983; Leijendekker & Hardveld, 1987).

The results obtained on whole muscles might be explained by the observation, that it has been shown hyperthyroidism reduces relaxation time whilst hypothyroidism prolongs relaxation time (Gold et al., 1970; Fitts et al., 1980, 1984; Johnston et al., 1980b; Nicol & Bruce, 1981; Nicol & Maybee, 1982; Everts, 1983) and since relaxation is mainly brought about by the re-uptake of Ca^{2+} into the SR. A reduction of relaxation time implies an increased rate of Ca^{2+} uptake into the SR whilst a prolongation implies the opposite. An increased rate of Ca^{2+} uptake might result in a lower cytosolic free Ca^{2+} concentration, which in turn would decrease the active tension as seen in hyperthyroidism. Whilst a decreased rate of Ca^{2+} uptake might result in a higher cytosolic free Ca^{2+} concentration, which in turn would increase the active tension as seen in hypothyroidism.

Evidence for effects of dysthyreosis on the Ca^{2+} transport function of SR is provided by experiments of several investigators. In hyperthyroidism an increased rate of Ca^{2+} uptake has been observed in soleus muscle (Kim et al., 1982; Nwoye et al., 1982) whereas in hypothyroidism a decreased rate of Ca^{2+} uptake is found in a preparation of all hind limb muscles (Fanburg, 1968) and in gastrocnemius muscle (Simonides & Hardveld 1985).

Alternatively the results on whole muscle preparations might be explained by activation of fewer muscle fibres in hyperthyroid fast muscles and activation of more muscle fibres in hypothyroid fast muscles

which would lead to a decreased and increased isometric tension respectively. This might be the case because, a reduction in the membrane potential and an increase in the threshold for the generation of the action potential have been observed in fast skeletal muscle of hyperthyroid rats (McCardle et al., 1977) whilst a decrease in the threshold for the generation of the action potential together with an increase in action-potential amplitude have been described to occur in fast muscles of hypothyroid rats (Grossie, 1978). According to several authors (McCardle et al., 1977; Grossie, 1978; Hofmann & Denys, 1972) the observed alterations in the excitability of the sarcolemma due to hypo- or hyperthyroidism might result in the activation of fewer muscle fibres in the hyperthyroid state and more muscle fibres in the hypothyroid state.

However the present results on single muscle fibres cannot be explained by either of the two explanations which may be valid in whole muscle preparations. The results suggest either an increase in the tension per cross bridge of hypo- fibres with respect to hyperthyroid fibres and/or number of strong cross bridges have increased.

Since MLC_2 phosphorylation has been shown to enhance post tetanic twitch tension in euthyroid skeletal muscles (Manning & Stull, 1982), it is possible that the higher tension in hypothyroid fibres is a consequence of a higher degree of MLC_2 phosphorylation in hypo- fibres as opposed to hyperthyroid fibres. A possible mechanism for increased MLC_2 phosphorylation could be for hypothyroidism to increase the activity of the myosin light chain kinase (MLCK) which catalyses the phosphorylation reaction.

However, Leijendekker & Hardveld (1987) showed MLC_2 phosphorylation to be similar between eu- and hypothyroid fast muscles and they observed no difference in the MLCK activity either.

Furthermore, steady-state isometric tension at saturating calcium concentrations has been shown to be unchanged by MLC_2 phosphorylation, indicating that tension is unaffected by phosphorylation (Persechini et al., 1985; Metzger et al., 1989). Therefore, a higher degree of MLC_2 phosphorylation is unlikely to account for the increased tension in hypothyroidism.

Alternatively, the number of strong cross bridges could have increased through an increase in contractile protein, but this does not seem to be the case. Firstly only mild treatments were used which did not significantly alter the normal growth and development of the animals. Secondly, for the contractile mass to have increased a significant difference in muscle weight between hyper- and hypothyroid muscles should be observed, but this was not the case (present study) and the fibre diameter would be expected to increase in hypothyroidism to reflect the higher contractile mass, but this was not observed either (present study). Another factor would be the phenotype of the fibre was not changed, as reflected by the histochemical results, suggesting only minor modifications in the contractile proteins which are known to be isomorphic with the exception of actin (Swynghedauw, 1986).

Finally, the increase in tension per cross bridge and/or number of cross bridges in hypothyroid muscle fibres could be due to a change in the MLC profile from fast to slow in the soleus and TFL muscle fibres. It is possible that the MLC profile could in some manner modulate the tension, as to how this might occur is not apparent. Interestingly, the observed higher P_o in euthyroid soleus fibres than TFL euthyroid fibres in the present study is consistent with the idea that the presence of slow light chains might enhance P_o.

Although the results on whole muscles have been explained on the basis of increased/decreased Ca^{2+} uptake or activation of more/less fibres, in view of the present results they would be better explained by an effect mostly on the contractile proteins. Firstly, the percentage increase/decrease observed on whole muscles is comparable to the present study which has used only mild forms of treatment. Therefore, if the effects were additive (e.g., increased intrinsic strength and decreased Ca^{2+} uptake) then a much larger % increase/decrease would be expected on whole muscles than that observed.

Secondly the increased/decreased Ca^{2+} uptake could be countered by an increased/decreased release of Ca^{2+} from the SR in the first instance, thus ensuring a similar active Ca^{2+} concentration around the contractile filaments in different thyroid states. In fact it has been reported (Fitts et al., 1980) that thyroid hormone induces a large proliferation of the SR with a simultaneous increase in Ca^{2+} pumps suggesting an increased amount of Ca^{2+} release which could be cancelled by the increased Ca^{2+} uptake, pointing to a similar active Ca^{2+} concentration around the contractile filaments. Furthermore, a larger release of Ca^{2+} by the SR is suggested by the finding that Ca^{2+} release after K^{+} depolarisation was significantly enhanced in soleus muscles of hyperthyroid rats (Hardveld & Claussen, 1984).

Thirdly, the activation of more/less fibres would not explain the results from the soleus muscle as the excitability of the sarcolemma membrane is not affected in dysthyreosis (Grossie, 1978).

The present results could be a partial explanation as to why the weakness in hypothyroidism is less prominent in comparison to the hyperthyroid situation (Ramsay, 1974). That is, it is the tension per cross bridge and/or number of cross bridges which have changed in addition to any other changes which might occur.

For both the soleus and TFL fibre measurements, V_{max} of the hyperthyroid fibres was significantly higher ($P < 0.05$) than hypothyroid fibres (Table 4.1).

To our knowledge, this is the first study on skinned single muscle fibres from hyper- and hypothyroid rats. It has shown a significant increase in V_{max} of hyper- fibres compared to hypothyroid fibres without a change in the fibre type, as identified histochemically. Furthermore, this difference in V_{max} due to the thyroid state occurs in both the soleus and TFL muscles.

Since skinned fibres were used, and since the composition of solutions bathing the contractile proteins were carefully controlled, any differences in contractile performance should be attributable to differences in the contractile apparatus. Measurements for V_{max} were made at maximally activating calcium concentrations. Thus contractile differences would be due to alterations in either myosin or actin rather than the regulatory proteins (tropomyosin and troponin). Since actin isoenzymes have not been demonstrated to vary among adult skeletal muscle types (Vanderckhove & Weber, 1979), the observed variations in V_{max} are most likely due to myosin variations which suggests that dysthyreosis induces a change in the isomyosins.

Secondly this increase has been shown to occur in the same fibre type, suggesting that myosin ATPase fibre typing does not correlate with small changes in V_{max} (only a small change was observed due to the same type of fibre being sampled, otherwise a 2-3 fold increase would have been observed for the soleus muscle fibres due to the FOG fibres being 2-3 times faster whereas in the fast muscle a smaller change (if any) would have been observed) or is not sensitive enough to detect these changes. However, the mostly likely explanation is an extension of the latter reason which is a change in the light chains of the fibres sampled.

This is primarily because it has been shown that the histochemical fibre typing of myosin ATPase activity depends on the predominant type of MHC (MHC may exist in the same fibre in more than one isoform, the histochemical reaction being dictated by the predominant isoform – Reiser et al., 1985a; Danieli-Betto, 1986; Staron & Pette, 1987a, b) but not on the MLC present in the fibre (Staron & Pette, 1987a, b). Indeed it has been demonstrated that the three types of fibres correspond to three different myosin heavy chains (Danieli-Betto et al., 1986) and these are coded by three different genes (Izumo et al., 1986). Furthermore it has been shown that V_{max} in fibres correlates highly with the MHC composition of the fibre (Reiser et al., 1985a, b; Edman et al., 1988; Sweeney et al., 1988) but it has also been shown that alkali light chains (Greaser et al., 1988; Sweeney et al., 1988) and the phosphorylatable light chains (Moss et al., 1982; Hofmann et al., 1990) have a modulatory effect on V_{max}. Moreover, a recent study (Lowey et al., 1993) has shown that both classes of myosin light chains are essential for physiological speeds of shortening.

Therefore, a tentative conclusion is mild dysthyreosis modulates V_{max} in the fibres by a change in the myosin light chain profile. Indeed changes in synthesis of specific myosin light chains have been reported in hypo- (Johnson et al 1980a, b) and hyperthyroidism (Ianuzzo et al., 1980; Nwoye et al., 1982).

This increase in the V_{max} without a change in the fibre type may explain why the changes in muscle contractile properties precede detectable effects on the distribution of fibre types (Nicol & Bruce, 1981). Nicol & Maybee (1982) attributed this effect on the reduced elasticity of the soleus muscle which could be due to the catabolic effects of thyroid hormones on connective tissue and the resultant increased turnover of collagen as indicated by an elevated urinary excretion of hydroxyproline

(Ingbar & Woebar, 1974). However, whether the light chains which seem to change would be changed within one week and whether relaxation time of the soleus (i.e. the effect which is detected the earliest – Nicol & Bruce, 1981) or the contraction time will be affected by changing light chains remains to be seen.

A tentative conclusion from the present results is that the contractile proteins were changed per se and specifically the isomyosins are in a continuous dynamic flux with different fibres being at different stages regarding protein turnover. However, when a transformation stimulus such as dysthyreosis is applied the first response of the fibre is to modulate the V_{max} by the myosin light chains which could alter the kinetics of interaction of the contractile proteins, but if the stimulus continues then the next step is to change the myosin heavy chains.

The effects on P_o and V_{max} for fibres from both muscle types were of similar magnitude which seems to contradict the results obtained on whole muscles by many investigators which suggest that fast muscle is less responsive than slow muscle. However, the present results could be due to the type of fast muscle used, but is highly unlikely because muscles composed mainly of FG fibres have been shown to be even less responsive than muscles composed mainly of FOG fibres (Winder & Holloszy, 1977; Winder et al., 1980; Sickles et al., 1987; Fitzsimons et al., 1990).

The most likely reason is that due to the sampling of the same type of fibres (and hence only minor modifications in contractile proteins), results of similar magnitude have been observed e.g., if the converted FOG fibres had been sampled a 2-3 fold difference would have been expected in the soleus whereas not a large difference would have been observed in the TFL muscle. Alternatively, the effects on the fast muscle

contractile proteins might be cancelled out by opposite effects on other structures/processes at the whole muscle level.

Finally, the issue of whether T_3 induces changes by a direct effect of thyroid hormones on muscle or by an indirect effect by altering neuronal activity and/or trophic chemical synthesis in the motorneuron cannot be resolved here. However, previous studies suggest it acts directly on the muscle to regulate the MHC phenotype (Nwoye & Mommaerts, 1981; Nwoye et al, 1982; Hall-Craggs et al., 1983).

In conclusion, mild dysthyreosis shows small but significant changes in P_o and V_{max} in the same type of fibres as identified histochemically from slow and fast muscles. These changes are probably related to the effects on the contractile proteins and specifically the myosin light chains.

CONTRACTILE PROPERTIES OF HYPER- /HYPOTHYROID SOLEUS AND TFL FIBRES AT 25 °C, pH 7.0

For both the soleus and TFL fibre measurements, the weight of the hyper- and hypothyroid animals was not significantly different from each other (Table 5.1). The core temperature of the animals was lower in hypo- than hyperthyroid animals, as is to be expected since hypothyroidism decreases the metabolic rate. The diameter of the fibres, within each muscle type, was similar in both thyroid states.

Table 5.1: Measurements of soleus and TFL muscle fibres from hyper- and hypothyroid rats.

	Hyperthyroid	Hypothyroid
	SOLEUS	
Rat weight (g)	376.75 ± 6.33 (N = 6)	367.70 ± 7.12 (N = 6)
Core temperature (°C)	37.17 ± 0.21 (6) *	36.25 ± 0.17 (6)
Fibre diameter (µm)	57.81 ± 1.05 (28)	56.25 ± 1.67 (27)
V_{max} 7.0 (L_oS^{-1})	4.93 ± 0.50 (16)	4.94 ± 0.33 (19)
	TFL	
Rat weight (g)	389.33 ± 2.19 (N = 3)	395.00 ± 2.65 (N = 3)
Core temperature (°C)	37.00 ± 0.00 (3)	36.67 ± 0.17 (3)
Fibre diameter (µm)	67.92 ± 2.52 (15)	69.28 ± 2.82 (18)
V_{max} 7.0 (L_oS^{-1})	12.17 ± 0.85 (13)	11.61 ± 0.64 (13)

* $P < 0.05$, hyper- vs. hypothyroid

MAXIMUM VELOCITY OF SHORTENING

The $V_{max\ 7.0}$ (i.e., V_{max} measured at the end of the T/pCa relationship) values were not significantly different between the two treated states, within each muscle type. However, a significant difference in V_{max} has been shown to exist in both muscle types (chapter 4). This suggests that after activity there is a significant decrease in the $V_{max\ 7.0}$ of hyperthyroid fibres irrespective of fibre type whereas there is no significant effect in the hypothyroid state. As to why there is this differential decrease in maximum velocity of shortening, this is not apparent but it could be a partial explanation for the fast fatigue observed in hyperthyroid patients (Ramsay, 1974).

As described below, it is unlikely that V_{max} was decreased due to a build up of metabolites and furthermore it is also unlikely to be due to light chain phosphorylation either, since light chain phosphorylation does not affect V_{max} (Persechini et al., 1985).

T/pCa RELATIONSHIPS

For the T/pCa relationships, as the Ca^{2+} concentration increased, larger tensions per unit CSA were observed in fibres, from both types of muscles, irrespective of thyroid status (Fig. 5.1). This modulation of tension can be simply related to the number of cross bridges. Since, for any calcium concentration the steady tension is proportional to the number of cross bridges which should be graded with partial activation as described by Brenner & Yu (1983).

Generally, for a given $[Ca^{2+}]$ hyperthyroid fibre tensions appeared larger than the hypothyroid fibre tensions, irrespective of muscle type (Fig. 5.1a,d). These tensions scaled relative to $P_{7.0}$ (i.e., to the maximum tension generated at the end of the T/pCa relationship, in a solution of

pCa 4.14, pH 7.0) were related to the pCa values as shown in Fig. 5.1b,e. For the soleus fibres, the T/pCa relationship was shifted to the right in the hyperthyroid state with respect to the hypothyroid state, indicating a reduction in the calcium sensitivity of tension development in the hyperthyroid state. Whereas, for the TFL fibres, the T/pCa relationship was shifted to the left in the hyperthyroid state with respect to the hypothyroid state, indicating an increase in the calcium sensitivity of tension development in the hyperthyroid state.

From the soleus fibre Hill plots (Fig. 5.1c), the calculated Ca^{2+} threshold for activation (Table 5,2), and the Ca^{2+} concentration required to obtain half maximal tension was higher (i.e. required more Ca^{2+}) in hyper- than in hypothyroid fibres. The Ca^{2+} concentration required to obtain maximal tension was similar in both hyper- and hypothyroid fibres. Moreover, a steeper T/pCa relationship is indicated by a higher n value for hyper- as oppose to hypothyroid soleus fibres, however, it was not significant. The ratio of hyper- to hypothyroid fibres for the value of n was 1.12.

Table 5.2: Summary of the calculations from the Hill plot data for fibres from hyper- and hypothyroid soleus muscles at pH 7.0. The n values are given with their 95% confidence limits.

	Hyperthyroid	Hypothyroid
pCa_{TH}	6.67	6.87
Free $[Ca^{2+}]$ (M)	2.14×10^{-7}	1.35×10^{-7}
pCa_{50}	5.80	5.89
Free $[Ca^{2+}]$ (M)	1.58×10^{-6}	1.29×10^{-6}
n	1.73 ± 0.10	1.54 ± 0.15

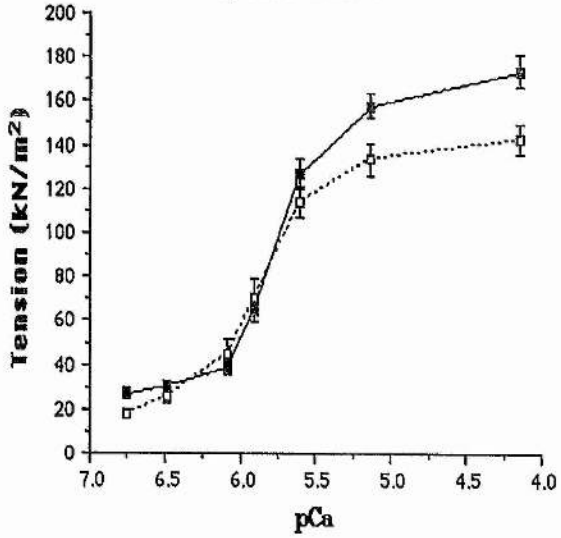
From the TFL fibre Hill plots (Fig. 5.1f), the calculated Ca^{2+} threshold for activation (Table 5.3), the Ca^{2+} concentration required to obtain half-maximal tension and the Ca^{2+} concentration required to obtain maximal tension was lower, (i.e. required less Ca^{2+}) in hyper- than in hypothyroid fibres. Moreover, a steeper T/pCa relationship is indicted by a higher n value for hyper- as oppose to hypothyroid TFL fibres, however, it was not significant. The ratio of hyper- to hypothyroid TFL fibres for n was 1.23.

Table 5.4: Summary of the calculations from the Hill plot data for fibres from hyper- and hypothyroid TFL muscles at pH 7.0. The n values are given with their 95% confidence limits.

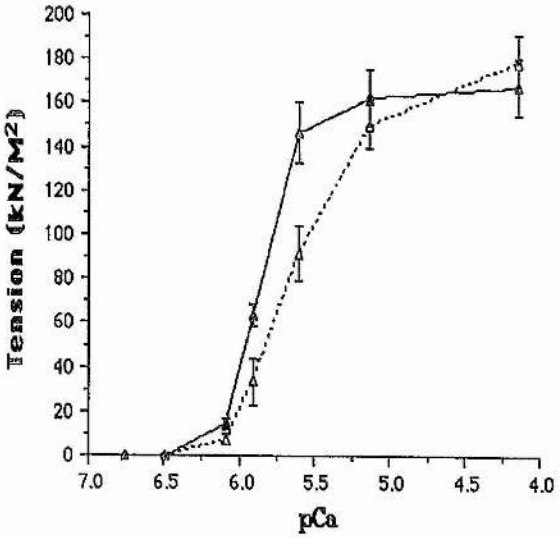
	Hyperthyroid	Hypothyroid
pCa_{TH}	6.39	6.35
Free $[Ca^{2+}]$ (M)	4.07×10^{-7}	4.47×10^{-7}
pCa_{50}	5.77	5.60
Free $[Ca^{2+}]$ (M)	1.70×10^{-6}	2.51×10^{-6}
n	2.44 ± 0.28	1.99 ± 0.10

Fig. 5.1: Isometric T/pCa relationships, Relative T/pCa relationships and average Hill plots of single skinned fibres from hyper- (—■—) and hypothyroid (·····□·····) soleus muscles, (a) to (c) respectively. And for hyper- (—▲—) and hypothyroid (·····△·····) TFL muscles, (d) to (f) respectively. Mean values ± SEM (which are indicated by the vertical bars or by the size of the symbols used) were obtained from 15 to 28 fibres.

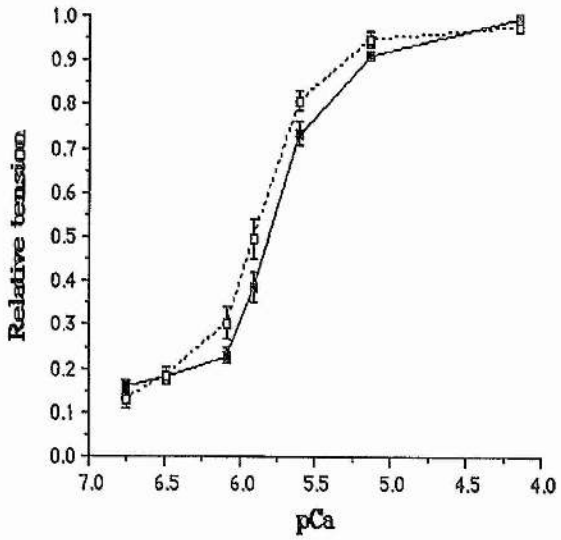
a) Soleus fibres



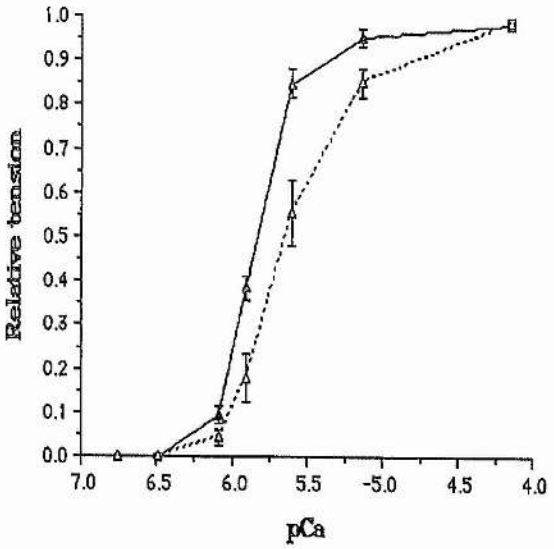
d) TFL fibres



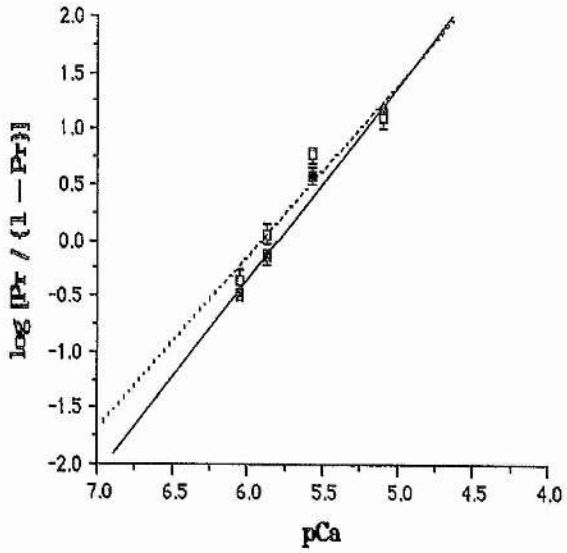
b) Soleus fibres



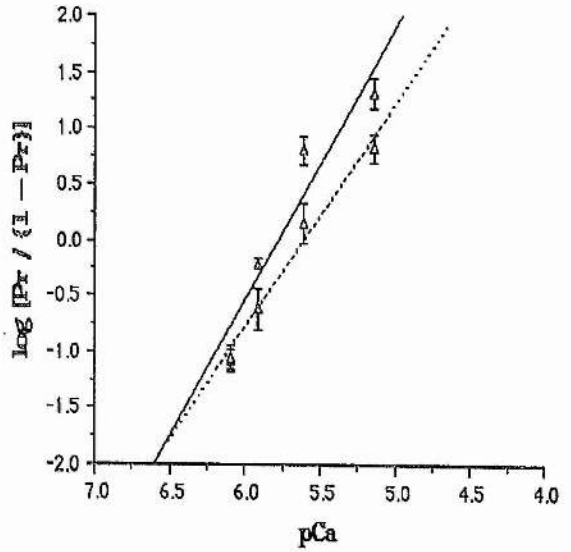
e) TFL fibres



c) Soleus fibres



f) TFL fibres



The results of the calcium activated tension responses for the soleus muscle fibres (steeper T/pCa relationship which is shifted to the right i.e., a lower Ca^{2+} sensitivity in hyper- than hypothyroid fibres) seem to be consistent with the idea that the sensitivity of the contractile apparatus converted from being of the slower type in hypothyroid fibres to being of the faster type in hyperthyroid fibres. This seems to happen even though histochemically the fibres were identified as being SO in both treated states. Therefore, this suggests that dysthyreosis induced changes in the Ca^{2+} regulatory proteins from slow to fast in the direction of hypo- to hyperthyroid, although histochemically the same type of fibres were identified. In other words the dynamic state of the fibres was shifted from slow to fast in the direction hypo- to hyperthyroid.

The results of the calcium activated tension responses for the TFL muscle fibres (steeper T/pCa relationship which is shifted to the left, i.e., a higher Ca^{2+} sensitivity in hyper- than hypothyroid fibres) also seem consistent with the idea that the contractile apparatus converted from the faster type in hyperthyroid fibres to the slower type in hypothyroid fibres, with only the FG fibres being sampled between the two different states. Although the shift in Ca^{2+} sensitivity was in the opposite direction to that found in the SO fibres. This can be reconciled as follows: in the hypothyroid TFL muscles the fibres convert from the FG to the FOG type whereas the same occurs in the hyperthyroid state but to a lesser extent, as seen by whole muscle histochemistry (present study). Therefore, although the fibres from the fast muscle in the present study in both treated states were of the FG type, it is reasonable to hypothesise that the underlying dynamic state of the Ca^{2+} regulatory protein isoforms had shifted to the FOG type in both states but the extent of this shift was larger in the hypo- as oppose to the hyperthyroid

state. Now, where authors have described differences in T/pCa relationships between FG and FOG fibres (Schachat et al., 1987; Greaser et al., 1988, Danieli-Betto, 1990), they have shown FG fibres to have steeper T/pCa relationship and higher Ca^{2+} sensitivity than FOG fibres. Therefore the suggestion is that in both treated states the shift is from FG to FOG with respect to the Ca^{2+} regulatory proteins but this shift is larger in the hyperthyroid state and hence the fibres are more akin to the FG type whereas in the hypothyroid state they are more akin to the FOG type and thus the observed results. It appears that different isoforms of Tn T are responsible for a higher Ca^{2+} sensitivity between the intermediate (FOG) and high velocity (FG) fibres (Greaser et al., 1988).

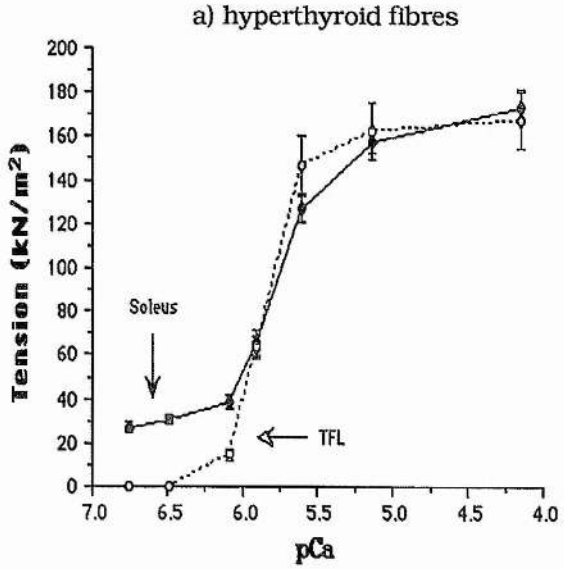
In short, the results have shown that the muscle fibres of the same histochemical type can generate heterogeneous T/pCa relationships, which are in agreement with those obtained by authors using histochemically identified fibres (Zeman & Woods, 1980; Donaldson 1984). First of all it shows histochemistry alone cannot distinguish between the different fibre types and alternative fibre types can be identified according to contractile function (Danieli-Betto, 1990; Wilson & Stephenson, 1990). The heterogeniety observed by these authors is not due to a transformation stimulus but the fact that fibres exist in various isomorphic forms with respect to the contractile and regulatory proteins. In fact it has been suggested that a dynamic continuum of fibre type exists which encompasses all fibre types (Staron et al, 1983; Staron & Pette, 1987a, b; Wilson & Stephenson, 1990).

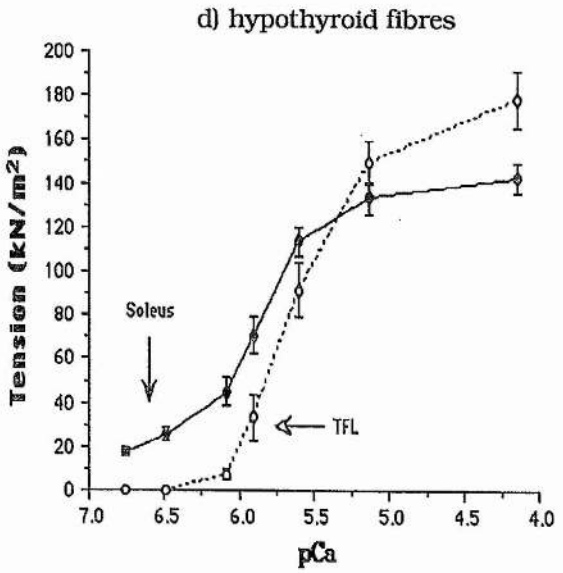
In the present study the heterogeniety is likely to be due to the effect of dysthyreosis on these isomorphic regulatory proteins and the myosin light chains, which shifts the dynamic state of the fibres to fast and slow type respectively in hyper- and hypothyroid fibres. It is thought that the fibres from the treated animals which are undergoing

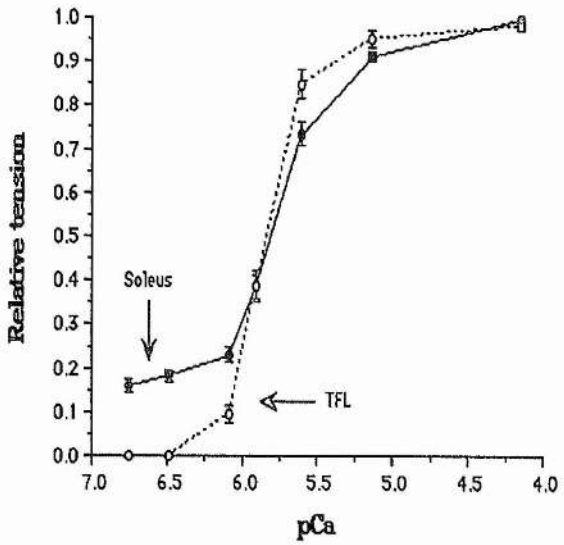
transformation are likely to have different proportions of slow and fast isoforms of the proteins and it has been shown that when both fast and slow isoforms of regulatory proteins and of MLC are present in a muscle fibre, calcium sensitivity is dictated by the fast isoforms (Danieli-Betto, 1990) which could be why a difference has been observed in the T/pCa relationships.

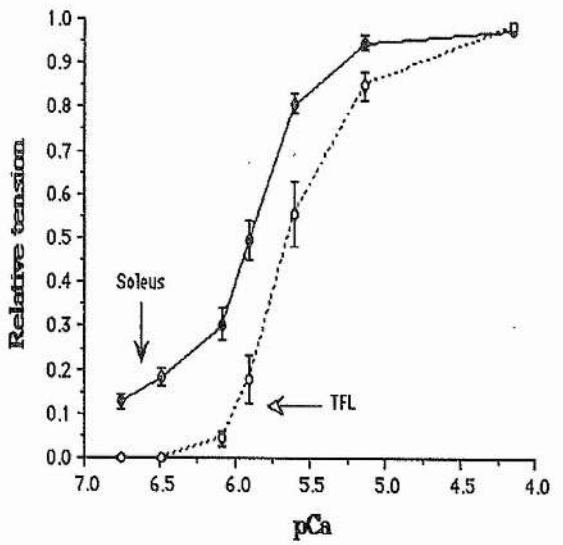
In addition, The T/pCa relationships (Fig. 5.2) showed that irrespective of the thyroid status soleus muscle fibres had a higher pCa_{TH} for tension development and a less steep T/pCa relationship than the TFL muscle fibres. These results are in general agreement with results of other authors who have described such differences between the Ca^{2+} activated tension responses of slow and fast muscle fibres obtained from euthyroid animals (Takagi & Endo, 1977; Stephenson & Forrest, 1980; Stephenson & Williams, 1981; Eddinger & Moss, 1987; Laszewski-Williams et al., 1989; Mounier et al., 1989), suggesting these differences remain even when the thyroid status of the animal is altered. This could be explained if dysthyreosis affected the contractile apparatus to a similar extent therefore inducing changes in the direction slow to fast for hypo- to hyperthyroid respectively.

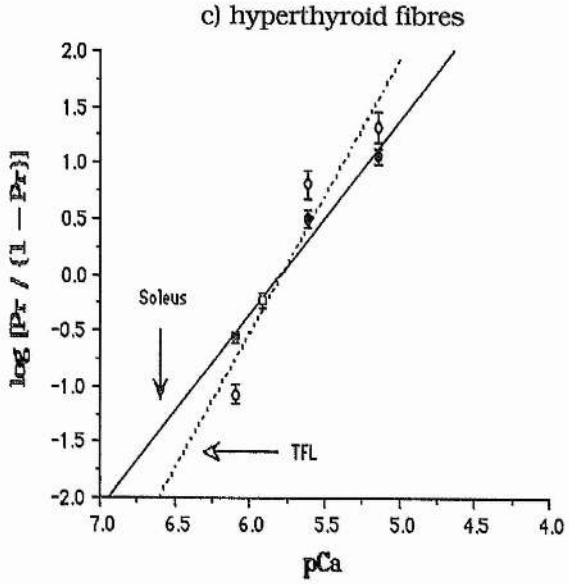
Fig. 5.2: Isometric and relative T/pCa relationships, and Hill plots of single skinned fibres from hyperthyroid soleus (—●—) and TFL muscles (·····o·····), (a) to (c) respectively . and for hypothyroid soleus (—●—) and TFL muscles (·····o·····), d) to f) respectively. Mean values ± SEM were obtained from 15 to 28 fibres.

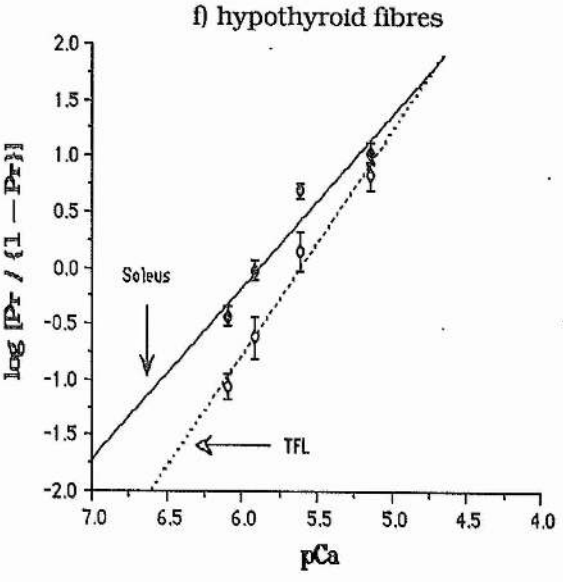

a) hyperthyroid fibres
Tension (kN/m²)
Soleus
TFL
pCa


d) hypothyroid fibres
Tension (kN/m²)
Soleus
TFL
pCa


b) hyperthyroid fibres
Relative tension
Soleus
TFL
pCa


e) hypothyroid fibres
Relative tension
Soleus
TFL
pCa


c) hyperthyroid fibres
log [Pr / (1 — Pr)]
Soleus
TFL
pCa


f) hypothyroid fibres
log [Pr / (1 — Pr)]
Soleus
TFL
pCa

However, with respect to the Ca^{2+} sensitivity, the difference in the hypothyroid state between soleus and TFL fibres is similar to that of untreated fibres but in the hyperthyroid state there is no difference between the two different fibre types. This can be understood by extension of the hypothesis outlined above. It is also reasonable to assume that the equilibrium of the SO fibres shifts to FOG in the hyperthyroid state and to left of SO fibres in the hypothyroid state (Fig. 9.1). Therefore, in hyperthyroid soleus and hyperthyroid TFL muscles the fibres sampled would be of a similar kind of FOG type with respect to the regulatory proteins and hence the similarity between the curves.

The following explanations for the different characteristics of the T/pCa relationships which apply to euthyroid muscle fibres are also likely to apply to hyper- and hypothyroid muscle fibres since the major effect of these treatments is to shift the dynamic state of the fibres to that resembling those of the fast or slow type respectively.

The presence of slow or fast isoforms of Tn C has been considered the main reason for the different calcium sensitivity for tension development and the steepness (i.e., co-opereativity) of the T/pCa relationship between slow and fast skeletal muscles (Moss et al., 1986; Babu et al., 1987; Gulati et al., 1988). Indeed it has been demonstrated that fast muscle Tn C has two low affinity calcium binding sites whereas slow skeletal muscle Tn C has only got one, in addition, both types of muscle have two high affinity Ca^{2+}–Mg^{2+} sites (Potter & Gergely, 1975).

However. many additional factors may contribute to the differential Ca^{2+} sensitivity of slow and fast muscle fibres and then to the T/pCa relationship. It has been shown that a greater Ca^{2+} sensitivity is associated with the presence of higher molecular weight isoforms of Tn

T (Greaser et al., 1988). Moreover, the isoforms of Tm and Tn I as well as the interactions between them may also affect Ca^{2+} sensitivity (Grabarek et al., 1983 1986; Schachat et al., 1987). MLC phosphorylation has also been shown to increase Ca^{2+} sensitivity (Sweeney & Stull, 1986, 1990). It has also been reported that when the actomyosin interaction is being activated, the activated cross bridges in turn affect Tn C structure and thus increase calcium sensitivity (Gordon et al., 1988). Furthermore, the affect of cross-bridges on the calcium activation of the thin filament may also be modulated by the regulatory MLC_2 (Metzger et al., 1989; Sweeney & Stull, 1990; Hofmann et al., 1990).

Our results do not allow discrimination between these possibilities, although the role of the Ca^{2+} regulatory proteins and the light chains is implicated.

A possible explanation given by Moss et al. (1986) to account for the differences in the steepness of the T/pCa relationships included the idea that interactions with adjacent tropomyosins molecules were necessary in the co-operative mechanism. According to Schachat et al. (1987), the responses of skeletal muscle fibres to Ca^{2+} were also determined by their composition in regulatory complexes TnT-Tm in the thin filament. Fast fibres from different muscles expressed different isoforms of TnT-Tm according to their speed and that difference influenced the steepness of the T/pCa relationship. So, it seemed that the influence of TnT on the end-to-end overlap of Tm was involved in the co-operativity mechanism.

A good correlation can be seen between the data showing that the calcium threshold for activation is lower in slow type than fast type fibres and the data of Dulhunty & Gage (1983) showing that the depolarisation threshold for the onset of contraction is lower in soleus

muscles of the rat. Since depolarisation triggers contraction via calcium release from the SR and the amount of released calcium is dependent on the amount of depolarisation (For review see Ruegg, 1986), it is reasonable to expect that the slow type fibres of the soleus, which need a lower calcium concentration for the onset of contraction, require a lower degree of depolarisation. This could be why there is an observed increase in the threshold of fast skeletal muscle of hyperthyroid rats and a decrease in the hypothyroid rats (McCardle et al., 1977; Grossie, 1978), since the whole system would be expected to work in co-ordination.

Dulhunty & Gage (1983) have also reported that the tension-voltage relationship has a greater slope in fast than in slow fibres and they pointed out the correlation between this observation and that of a steeper T/pCa relationship and a subsequent larger n value in fast than slow fibres. According to Dulhunty & Gage (1983), these differences may be attributed to differences in the excitation-contraction coupling process, or in other subsequent steps.

MAXIMAL ISOMETRIC TENSION

For the soleus muscles, the maximal isometric tension at pH 7.0, elicited by a pCa 4.14 solution (Fig. 5.1b) at the end of the T/pCa relationship was recorded and equal to 46.46 ± 2.21 mg (28) in the hyperthyroid soleus fibres and 36.74 ± 2.70 mg (27) in the hypothyroid soleus fibres. The difference between hyper- and hypothyroid fibres being significant ($P < 0.05$). When expressed per unit cross-sectional area ($P_{7.0}$) these values became respectively 173.39 ± 7.36 kN/m^2 and 142.32 ± 6.75 kN/m^2 in hyper- and hypothyroid fibres, the difference also being significant ($P < 0.05$).

For the TFL muscles, the maximal isometric tension at pH 7.0, elicited by a pCa 4.14 solution (Fig. 5.1e) at the end of the T/pCa relationship was equal to 63.27 ± 7.68 mg (15) in the hyperthyroid TFL fibres and 68.89 ± 5.89 mg (18) in the hypothyroid TFL fibres. The difference between hyper- and hypothyroid fibres being non significant. When expressed as $P_{7.0}$ these values became respectively 167.34 ± 12.78 kN/m^2 and 177.92 ± 12.84 kN/m^2 in hyper- and hypothyroid fibres, the difference also being non significant.

An apparent anomaly seems to exist between the observed P_o and $P_{7.0}$ values for the soleus muscle fibres. When P_o was measured the hypothyroid fibres had significantly higher values than hyperthyroid fibres whereas for the $P_{7.0}$ measurements the opposite was observed. The reason for this discrepancy is not apparent, however it is unlikely to be due to biological variability, which could be a possibility since observations were made on different fibre populations. This is mainly because this discrepancy was observed in both the soleus and to a lesser extent in the TFL muscle fibres. Moreover, $V_{max\ 7.0}$ values were also affected (see above). Therefore, the different method of measurement of these two parameters is implicated.

Essentially, the only difference in the way these measurements were made was that, P_o and V_{max} of a fibre were measured without any prior activity whilst $P_{7.0}$ and $V_{max\ 7.0}$ of a fibre were measured at the end of the T/pCa relationship measurements.

Therefore, a possible explanation for this discrepancy could be a general deterioration of the fibres with time and activity. However, this is unlikely, firstly because when comparing P_o with $P_{7.0}$ the latter value was higher than the former. And secondly, there is no apparent reason for a differential effect of time between hypo- and hyperthyroid fibres.

Another possible reason could be an increase in the build up of metabolites e.g., P_i and H^+ in the middle of the fibre with activity. However this is also unlikely as the ionic environment was controlled and the relaxation procedure would neutralise the effects of the metabolites. i.e., enough time to diffuse out and be buffered.

It is possible that MLC_2 phosphorylation could account for the discrepancy observed. In this respect, MLC_2 phosphorylation has been observed to increase maximal tension under fatigue conditions (Godt & Nosek, 1989). Therefore, the suggestion is that with prior activity MLC_2 phosphorylation could occur. Furthermore, a higher degree of MLC_2 phosphorylation would have to occur in hyper- than hypothyroid fibres, assuming the same amount of MLC_2 phosphorylation results in equal increase in tension in both hypo- and hyperthyroid fibres. Supporting evidence, for a higher degree of MLC_2 phosphorylation in hyper- than hypothyroid fibres comes from Leijendekker & Hardveld (1987). They found a higher degree of MLC_2 phosphorylation in eu- than hypothyroid muscles after activity suggesting a relatively decreased tension in hypothyroid muscles.

The MLC_2 could be phosphorylated by the repetitive nature of the protocol, which is analogous to the repetitive stimuli at low frequencies, which has shown to result in phosphorylation of MLC_2, probably due to small but significant amount of MLCK activated with each stimulation (Stull et al., 1982). However, whether significant MLC_2 phosphorylation would occur in the absence of added MLCK and calmodulin is debatable.

Finally it is possible, that prior activity induces very small changes in the three dimensional arrangement of the regulatory and contractile proteins in such a manner as to account for the differential results.

CONTRACTILE PROPERTIES OF HYPER-/HYPOTHYROID SOLEUS AND TFL FIBRES AT 25 °C, pH 6.6

For the soleus fibre measurements, the weight and the core temperature of the hyperthyroid animals was significantly higher (15%) and lower (4%) respectively than the hypothyroid animals (Table 6.1).

Table 6.1: Measurements of soleus and TFL muscle fibres from hyper- and hypothyroid rats at pH 6.6. $V_{max\ 6.6}$ was measured at pCa 4.10, pH 6.6, at the end of the pH 6.6 T/pCa relationship. $V_{max\ 7.0}$ was measured at pCa 4.14, pH 7.0, after having obtained $V_{max\ 6.6}$ in the same fibre.

	Hyperthyroid	Hypothyroid
	SOLEUS	
Rat weight (g)	429.83 ± 10.15 (N = 6) +	373.33 ± 6.01 (N = 6)
Core temperature (°C)	37.58 ± 0.20 (6) +	36.00 ± 0.26 (6)
Fibre diameter (µm)	57.92 ± 1.50 (28)	57.42 ± 1.61 (24)
$V_{max\ 6.6}$ (L_0S^{-1})	2.98 ± 0.23 (24)	2.81 ± 0.19 (22)
$V_{max\ 7.0}$ (L_0S^{-1})	4.64 ± 0.41 (22)	4.50 ± 0.46 (19)
	TFL	
Rat weight (g)	417.67 ± 14.40 (N = 3) *	350.60 ± 18.29 (N = 5)
Core temperature (°C)	37.17 ± 0.17 (3)	36.80 ± 0.26 (5)
Fibre diameter (µm)	68.47 ± 2.87 (11)	67.34 ± 1.69 (20)
$V_{max\ 6.6}$ (L_0S^{-1})	8.51 ± 1.21 (10)	7.91 ± 0.79 (19)
$V_{max\ 7.0}$ (L_0S^{-1})	11.17 ± 0.99 (8)	12.75 ± 1.20 (16)

* $P < 0.05$, + $P < 0.005$, hyper- vs. hypothyroid

For the TFL fibre measurements, the weight of the hyperthyroid animals was significantly higher (19%) but, the core temperature was only slightly (1%) higher than the hypothyroid animals, This non significant change in the core temperature is probably a reflection of a smaller number of animals used, rather than dysthyreosis having no

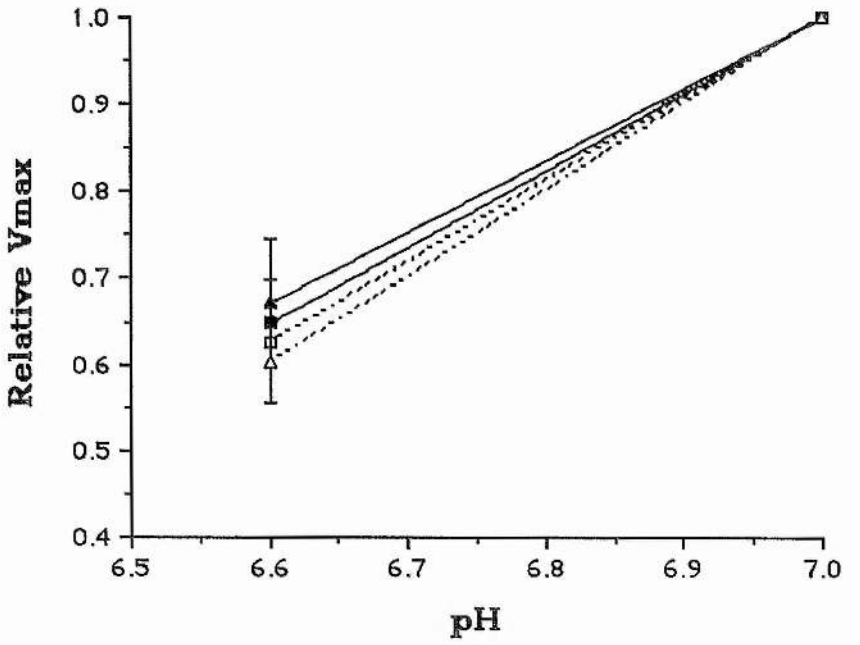
effect on the core temperature. It must be pointed out that the hypothyroid animals would be expected to have a lower core temperature than the hyperthyroid animals. Whereas, the core temperature of control and hyperthyroid animals would be expected to be similar. All the fibres sampled between the two different thyroid states, within each muscle type, had similar diameters.

EFFECT OF pH UPON V_{max}

For the hyperthyroid soleus fibres (Table 6.1), $V_{max\ 7.0}$ was 56% higher ($P < 0.005$, using two-tailed paired t-test) compared to $V_{max\ 6.6}$ and for the hypothyroid soleus fibres, $V_{max\ 7.0}$ was 60% higher ($P < 0.005$, using two-tailed paired t-test) compared to $V_{max\ 6.6}$. For the hyperthyroid TFL fibres, $V_{max\ 7.0}$ was 31% higher ($P < 0.05$, using two-tailed paired t-test) than $V_{max\ 6.6}$ and for the hypothyroid TFL fibres, $V_{max\ 7.0}$ was 61% higher ($P < 0.005$, using two-tailed paired t-test) than $V_{max\ 6.6}$.

Plotting relative V_{max} (i.e., dividing $V_{max\ 6.6}$ by $V_{max\ 7.0}$) against pH (Fig. 6.1), shows that relative V_{max} is depressed in the direction hyper- TFL > hyper- soleus > hypo- soleus > hypo- TFL. However, neither of these values are significantly different from each other.

Fig. 6.1: Variation with pH of V_{max} in hyperthyroid soleus (——■——), hypothyroid soleus (·····□·····), hyperthyroid TFL (——▲——) and hypothyroid TFL (·····△·····) single skinned fibres. Relative V_{max} values were obtained by dividing V_{max} 6.6 by V_{max} 7.0 in that same fibre. Values are the mean (± SEM) of 7 to 20 fibres.



Therefore, pH 6.6 depresses V_{max} in all four situations to a similar extent (i.e., 33 – 40%). These results are in qualitative agreement with results obtained by previous investigators from euthyroid animals (Luney & Godt, 1987; Metzger & Moss, 1987; Cooke et al., 1988). Although, Metzger & Moss (1987), found V_{max} of fast muscle fibres to be depressed significantly more than slow muscle fibres, that was only when they reduced the pH down to pH 6.2, whereas when the pH was reduced to pH 6.5, they found V_{max} to be depressed to a similar degree in both slow and fast muscle fibres.

A biphasic response of the slack test data (i.e., the plot of distance vs. time may be fitted with two straight lines, an initial high velocity phase and a second lower velocity phase. This latter phase has been attributed to a sub population of cross bridges which at low levels of thin filament activation exhibit a reduced rate constant for detachment,

thereby presenting an internal load which opposes further shortening (Moss et al., 1986)] which has been reported to occur at sub maximal Ca^{2+} activation (Moss et al. 1986) was not responsible for the decrease in V_{max} at lower pH in the present study, because the slack test data was best fitted by one straight line (see methods). Moreover, the decrease in V_{max} at lower pH was not due to a decrease in tension at lower pH, because it has been shown that V_{max} remains unchanged when it is measured at a similar tension at either pH 7.0 or pH 6.2 (Metzger & Moss, 1987).

One way in which H^+ ions could reduce V_{max} would be to decrease myosin ATPase activity (Barany, 1967). Consistent with this idea, the sigmoidal relationship between ATP hydrolysis and free $[Ca^{2+}]$ in myofibrils from rabbit muscle has been shown to be shifted to higher $[Ca^{2+}]$ and to become depressed as pH was lowered from 7.0 to 6.0 (Portzehl et al., 1969). Furthermore, V_{max} and ATPase activity of fibres has been shown to be reduced to a similar extent (Cooke et al., 1988). Low pH, presumably depresses ATPase activity by a direct inhibitory effect of H^+ ions on the ATPase thus slowing the cross bridge cycle rate.

Another possible explanation may be found in the observation that as pH is reduced myofilament lattice becomes compressed (April et al., 1972), thus this might alter cross bridge interaction with actin and decrease V_{max} (Metzger & Moss, 1987).

EFFECT OF pH UPON Ca^{2+} SENSITIVITY

Generally, for any given calcium concentration, fibres from soleus and TFL muscles, generated significantly higher tensions at pH 7.0 than at pH 6.6, irrespective of thyroid status (Figs. 6.2 & 6.3).

For the hyper- and hypothyroid soleus fibres (Fig. 6.2), when the tensions obtained at each pH were scaled relative to the maximum tension obtained (in the same fibre) at the same pH, the relative T/pCa relationships were shifted to the right at pH 6.6 with respect to pH 7.0. This shift was more apparent when the tensions obtained at each pH were scaled relative to the tension (in the same fibre) at pCa 4.14, pH 7.0,

Therefore, the effect of acidic pH on the soleus fibres was to reduce Ca^{2+} sensitivity (i.e. a higher free $[Ca^{2+}]$ was required to obtain the same tension when the pH was lower) irrespective of the thyroid status, which is similar to results reported by investigators using euthyroid soleus fibres (Donaldson & Hermansen, 1978; Donaldson, 1984; Metzger & Moss, 1987; Chase & Kushmerick, 1988). In addition, this shift was more pronounced in hyper- than hypothyroid soleus fibres, thus, this is consistent with the idea that the shift in the dynamic equilibrium of the fibres was merely from slow to fast in the direction of hypo- to hyperthyroid, since it has been reported that slow fibres are more resistant to the effects of acidic pH in reducing Ca^{2+} sensitivity than fast fibres (Donaldson, 1984). However, the results obtained for TFL fibres do not seem to fit within this hypothesis (see below).

The basis for pH modulation of the Ca^{2+} sensitivity of tension is unknown for certain but several possibilities have been proposed.

Fig. 6.2: Effect of pH on single skinned fibres from hyperthyroid and hypothyroid soleus muscles. Mean values (± SEM) at pH 7.0, (—■—) and at pH 6.6 (- - -□- - -) for hyperthyroid fibres and at pH 7.0, (—●—) and at pH 6.6, (- - -○- - -) for hypothyroid fibres were obtained from 19 to 28 fibres. In the fibres studied at pH 6.6, the maximum tension was always achieved at a free $[Ca^{2+}]$ well below pCa 4.10, and higher calcium led to significantly lower tension. Thus data for these fibres is scaled relative to the actual maximum tension obtained.

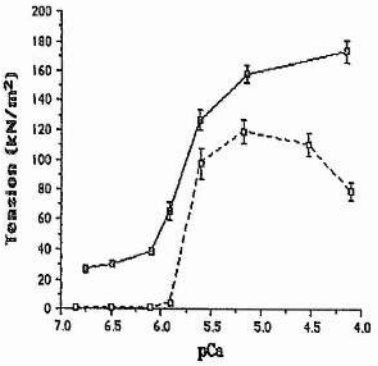
a) & e) Isometric T/pCa relationships.

b) & f) Relative T/pCa relationships. For the pH 6.6 T/pCa curve, the isometric tension at each $[Ca^{2+}]$ at pH 6.6, was scaled relative to the maximum tension generated in the same fibre at pH 6.6 ($P_{6.6}$).

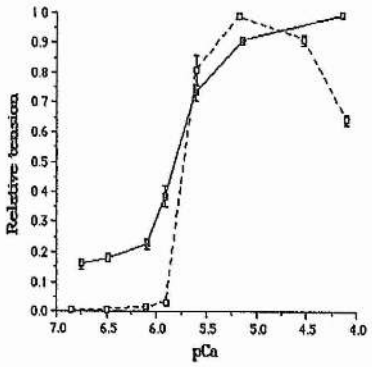
c) & g) Relative T/pCa relationships. For the pH 6.6 T/pCa curve, the isometric tension at each $[Ca^{2+}]$ at pH 6.6, was scaled relative to the maximum tension generated in the same fibre at pH 7.0 ($P_{7.0}$).

d) & h) Average Hill plots of individual relative ($P_{6.6}$) T/pCa relationships.

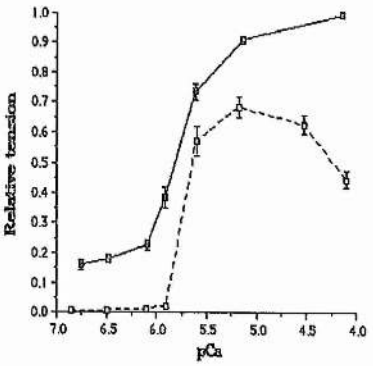
a) hyperthyroid Soleus



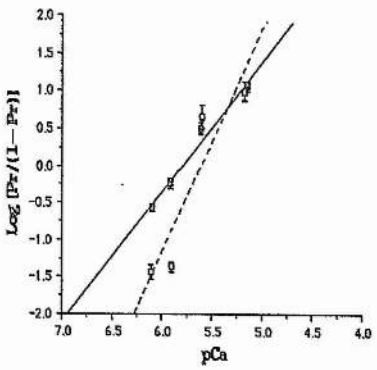
b) hyperthyroid Soleus



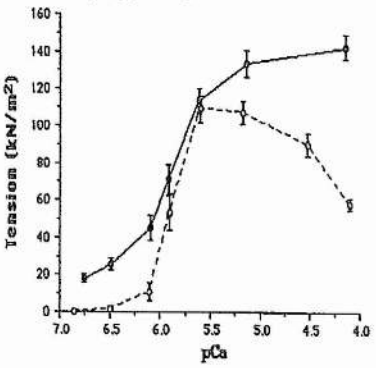
c) hyperthyroid Soleus



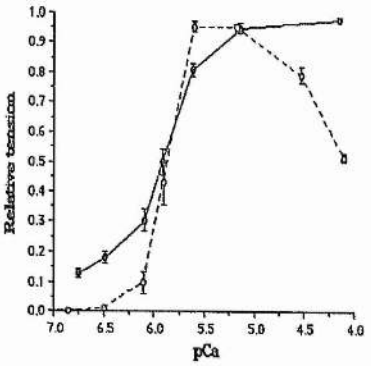
d) hyperthyroid Soleus



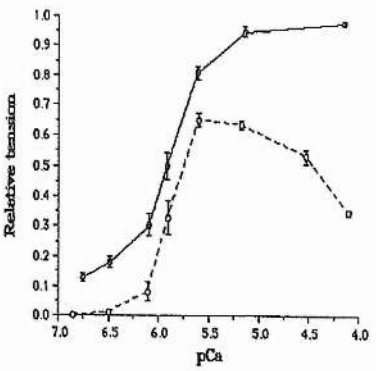
e) hypothyroid Soleus



f) hypothyroid Soleus



g) hypothyroid Soleus



h) hypothyroid Soleus

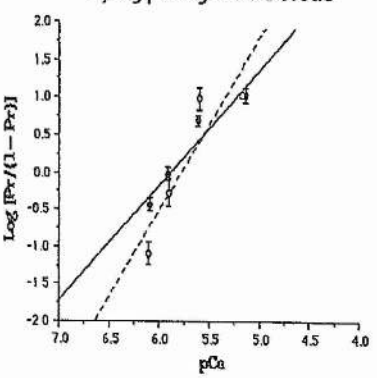


Fig. 6.3: Effect of pH on single skinned fibres from hyperthyroid and hypothyroid TFL muscles. Mean values (± SEM) at pH 7.0, (——●——) and at pH 6.6 (- - -△- - -) for hyperthyroid fibres and at pH 7.0, (——◇——) and at pH 6.6, (- - -◇- - -) for hypothyroid fibres were obtained from 8 to 18 fibres. In the fibres studied at pH 6.6, the maximum tension was always achieved at a free $[Ca^{2+}]$ well below pCa 4.10, and higher calcium led to significantly lower tension. Thus data for these fibres is scaled relative to the actual maximum tension obtained.

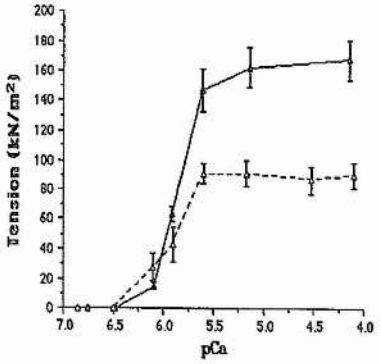
a) & e) Isometric T/pCa relationships.

b) & f) Relative T/pCa relationships. For the pH 6.6 T/pCa curve, the isometric tension at each $[Ca^{2+}]$ at pH 6.6, was scaled relative to the maximum tension generated in the same fibre at pH 6.6 ($P_{6.6}$).

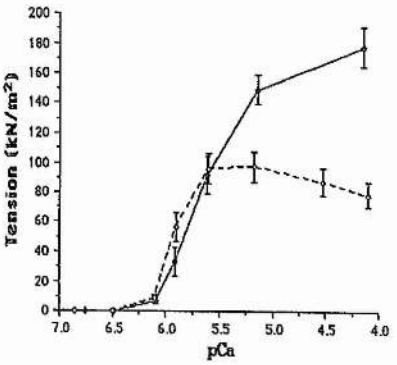
c) & g) Relative T/pCa relationships. For the pH 6.6 T/pCa curve, the isometric tension at each $[Ca^{2+}]$ at pH 6.6, was scaled relative to the maximum tension generated in the same fibre at pH 7.0 ($P_{7.0}$).

d) & h) Average Hill plots of individual relative ($P_{6.6}$) T/pCa relationships.

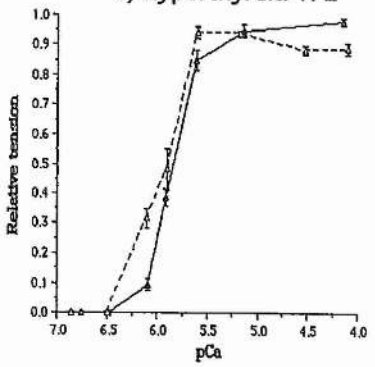
a) hyperthyroid TFL



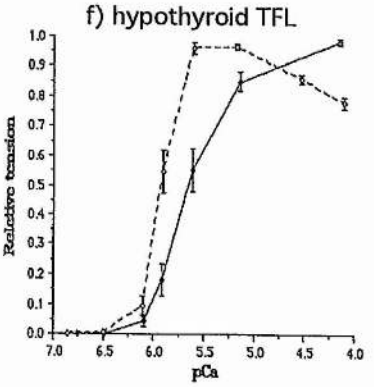
e) hypothyroid TFL



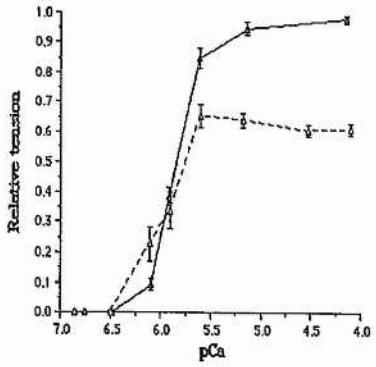
b) hyperthyroid TFL



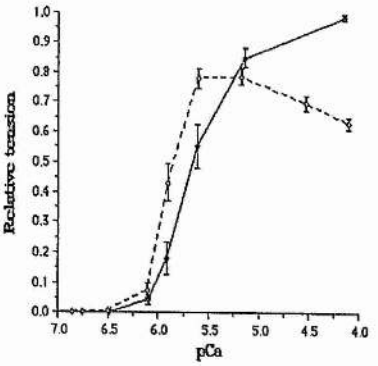
f) hypothyroid TFL



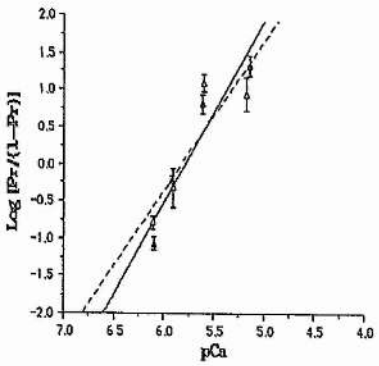
c) hyperthyroid TFL



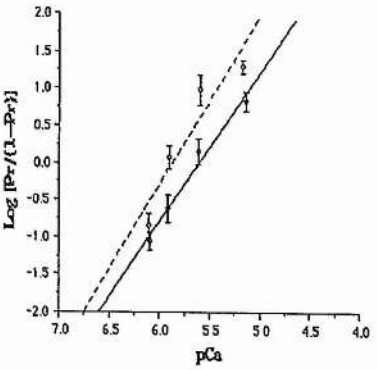
g) hypothyroid TFL



d) hyperthyroid TFL



h) hypothyroid TFL



The effect on Ca^{2+} sensitivity is thought to arise from competition between H^+ and Ca^{2+} at the thin filament with H^+ acting either directly at TnC (Robertson & Kerrick, 1979) or indirectly by altering the net charge on the thin filament (Godt, 1981). Godt proposed an electrostatic model to account for the H^+ ion-induced shift to the right. He argued that since contractile proteins bear a net negative charge at pH 7.0 the resultant electrostatic field about the myofilaments should attract cations and repel anions. Lowering the pH would reduce the net negative charge since the isoelectric points for these proteins are around pH 5.5, thereby changing the field about the myofilaments and decreasing the effective $[Ca^{2+}]$ near the thin filament.

Both the H^+/Ca^{2+} competition model and Godt's electrostatic model would certainly account for the reduction in Ca^{2+} sensitivity at low pH; however, they do not account for the reduction in maximum tension at low pH, since it should be possible to overcome the exclusion of Ca^{2+} from the filament lattice by raising the $[Ca^{2+}]$. This was not observed in this and many other studies (e.g., Fabiato & Fabiato, 1978; Metzger & Moss, 1987), where T/pCa relationships at low pH clearly reached a plateau or declined when $[Ca^{2+}]$ was increased to saturating levels.

Alternatively, since the presence of rigor cross bridges has been shown to enhance the binding of Ca^{2+} by TnC in regulated thin filaments (Bremel & Weber, 1972), and in addition, there is evidence to suggest that the cycling cross bridges also serve to activate, in a co-operative manner, regions of the thin filament by enhancing the binding affinity for Ca^{2+} (Guth & Potter, 1987). Then part of the decrease in Ca^{2+} sensitivity could be the result of a reduction in co-operative activation of the thin filament by attached strong cross bridges.

A decreased number of strong cross bridges is suggested by the finding of Metzger & Moss (1990a) who have shown that Ca^{2+} sensitivity of relative stiffness of a single fibre is reduced at low pH. Moreover, the Ca^{2+} sensitivity of the Ca^{2+} sensitive cross bridge transition from weak to strong cross bridges (Fig. 1.1, step 5) has been shown to be reduced (Metzger & Moss, 1990b). Therefore at low pH, cross bridges with weak binding would accumulate.

While the above mechanism could account for part of the reduction in the Ca^{2+} sensitivity to low pH it is likely other factors are involved as well, such as the ability of H^+ ions to reduce the affinity of Ca^{2+} binding sites on TnC (Blanchard et al., 1984; Kentish & Palmer, 1989), and possible protein–protein interactions, involving TnT, TnI and tropomyosin.

These other sites are also likely to account for the observed differential effect of H^+ ions on the Ca^{2+} sensitivity of hyper- and hypothyroid fibres, which cannot be readily explained in terms of decreased number of cross bridges and/or decreased tension per cross bridge. In this respect, there is evidence to suggest that the tension per cross bridge attachment is similarly depressed in slow and fast fibres (Metzger & Moss, 1990a) and H^+ ions decrease the rate constant governing the transition of weak to strong cross bridges to a similar extent in both slow and fast fibres (Metzger & Moss, 1990b).

In short, many factors (individually and/or in combination) are able to influence the position and the steepness of the T/pCa relationship, thus all these sites are possible targets to be modulated by H^+ ions.

The soleus fibre Hill plots (Fig. 6.2), show that when pH was decreased from 7.0 to 6.6, the free [Ca^{2+}] required to obtain pCa_{TH} increased by a factor of 3.63 in hyperthyroid soleus fibres as compared to a factor of 2.88 in hypothyroid soleus fibres (Table 6.2). The free [Ca^{2+}] required to obtain pCa_{50} increased by a factor of 1.58 in hyperthyroid soleus fibres as compared to a factor of 1.32 in hypothyroid soleus fibres. The Hill coefficient, n also increased to a larger extent in hyper- as opposed to hypothyroid fibres, These results show that the effect of reducing pH was more pronounced in hyper- than hypothyroid soleus fibres.

Table 6.2: Summary of Hill data of single skinned soleus fibres from hyper- and hypothyroid rats which have been analysed (in different fibre preparations) in various Ca^{2+} activating solutions of pH 7.0 and pH 6.6.

	Hyperthyroid		Hypothyroid	
	pH 7.0	pH 6.6	pH 7.0	pH 6.6
pCa_{TH}	6.67	6.11	6.87	6.41
pCa_{50}	5.80	5.60	5.89	5.77
n	1.73 ± 0.10 +	2.95 ± 0.43	1.54 ± 0.15 *	2.31 ± 0.38
ratio of n	1.71		1.50	

+ $P < 0.005$, pH 7.0 vs. pH 6.6 (hyper-); * $P < 0.05$, pH 7.0 vs. pH 6.6 (hypothyroid)

The increase seen in the Hill coefficient, n, in the soleus fibres at acidic pH, irrespective of thyroid status was also observed by Metzger & Moss (1987) in euthyroid soleus fibres. The increase in the Hill coefficient, n is probably due to a reduction in the number of active thin filaments, as described below, and the more pronounced effects on hyper- compared to hypothyroid fibres is in keeping with the hypothesis that larger the decrease in Ca^{2+} sensitivity the larger the increase in the Hill coefficient, n.

Although, the Hill coefficient, n, which is a measure of co-operativity, was observed to increase, whilst it is being suggested that Ca^{2+} sensitivity decreased due to a decrease in co-operative activation by the strong cross bridges. This apparent anomaly, can be explained by the hypothesis that, at decreased calcium sensitivity co-operative strong cross bridge attachment dominates thin filament activation. That is, as the number of strong cross bridges decreases; then the number of active thin filament units decreases for a given $[Ca^{2+}]$, and so isometric tension and calcium sensitivity both decrease. With a low calcium sensitivity, thin filament activation will be largely limited by the binding of strong cross bridges which is highly co-operative (Trybus & Taylor, 1980; Greene & Eisenberg, 1980), so the Hill coefficient, n increases.

For the hyper- and hypothyroid TFL fibres, when the tensions obtained at each pH were scaled relative to the maximum tension obtained (in the same fibre) at the same pH, the relative T/pCa relationships were shifted to the left at pH 6.6 with respect to those T/pCa relationships obtained at pH 7.0. This shift was still apparent in the hyper- and hypothyroid TFL fibres when the tensions obtained at each pH were scaled relative to the tension (in the same fibre) at pCa 4.10, pH 7.0.

Thus, in sharp contrast to the soleus fibres (see above), the effect of acidic pH on the TFL fibres was to increase Ca^{2+} sensitivity (i.e. a lower free $[Ca^{2+}]$ was required to obtain the same tension when the pH was lower) irrespective of the thyroid state, which is in contradiction to the results obtained from fast fibres in the euthyroid state (Metzger & Moss, 1987; Cooke et al., 1988; Godt & Nosek, 1989).

The slight increase observed in the sub maximal tensions at most calcium concentrations at low pH (Fig. 6.3), irrespective of the thyroid

status, could explain the increase in calcium sensitivity in as much as strong cross bridges enhance co-operative interactions. This would also explain the lower Hill coefficient, n values (Table 6..3). That is, the number of strong cross bridges increases, so for a given $[Ca^{2+}]$, the number of active thin filament units increases, and so isometric tension and calcium sensitivity both increase. With a high calcium sensitivity, activation of the thin filament will be mainly limited by Ca^{2+} binding, which is only slightly co-operative (Gabarek et al., 1983; Zot & Potter, 1987), and so the Hill coefficient, n decreases.

As to how H^+ ions could increase the sub maximal tensions, this is not apparent, but the possibility arises that dysthyreosis changed the myosin light chains and regulatory proteins in such a manner as to enhance Ca^{2+} sensitivity at low pH.

Finally, the main reason for the TFL fibre results is most probably due to the biological variability of the fibres, especially since the results were calculated from regression lines that were not significantly different. In addition, of all the reports on the effect of pH on euthyroid fast fibres, none have reported an increase in calcium sensitivity.

The TFL fibre Hill plots (Fig. 6.3), show that when pH was decreased from 7.0 to 6.6, the free $[Ca^{2+}]$ required to obtain pCa_{TH} decreased by a factor of 0.69 in hyperthyroid TFL fibres as compared to a factor of 0.66 in hypothyroid TFL fibres (Table 6.3). The free $[Ca^{2+}]$ required to obtain pCa_{50} also decreased by a factor of 0.91 in hyperthyroid soleus fibres as compared to a factor of 0.55 in hypothyroid soleus fibres.

The Hill coefficient, n decreased in hyperthyroid TFL fibres by 0.82-fold whereas it increased in hypothyroid TFL fibres by 1.14-fold., when pH was decreased from 7.0 to 6.6 (It must be borne in mind that these

results were calculated from regression lines which were not significantly different). This differential effect of n might be explained by the observation that Metzger & Moss (1987) found n to increase in slow and decrease in fast euthyroid fibres, and since as outlined earlier the dynamic equilibrium of the fibres shifts from slow to fast in the direction hypo- to hyperthyroid, then the hypothyroid TFL fibres would be expected to have an increased n value.

In both thyroid states of the TFL fibres, the sensitivity of the contractile apparatus increased when pH was reduced to pH 6.6 but the increase was larger in hypo- than hyperthyroid fibres.

Table. 6.3: Summary of Hill data of single skinned fibres from hyper- and hypothyroid TFL muscles which have been analysed (in different fibre preparations) in various Ca^{2+} activating solutions of pH 7.0 and pH 6.6.

	Hyperthyroid		Hypothyroid	
	pH 7.0	pH 6.6	pH 7.0	pH 6.6
pCa_{TH}	6.39	6.55	6.35	6.53
pCa_{50}	5.77	5.81	5.60	5.86
n	2.44 ± 0.28	2.01 ± 0.39	1.99 ± 0.10	2.26 ± 0.29
ratio of n	0.82		1.14	

It must be pointed out that Metzger & Moss (1987) used two best-fit straight lines rather than one, for the construction of Hill plots. As a consequence, two Hill coefficients were obtained by them for each plot: n_1 which corresponded to the slope of the fitted line for P_r greater than about 0.5, this remained unchanged in both slow and fast fibres when pH was reduced. And n_2 which corresponded to the slope of the fitted line P_r less than about 0.5, this increased in slow and decreased in fast fibres. However, in the present study the data were best fitted by one straight line.

In short, the differential effect of Ca^{2+} sensitivity between hypo- and hyperthyroid soleus fibres is consistent with the Ca^{2+} sensitivity differences obtained in euthyroid slow and fast fibres. However, the results (non-significant) obtained between hyper- and hypothyroid TFL fibres are at variance with observed euthyroid comparisons of slow and fast fibres.

EFFECT OF pH ON MAXIMUM TENSION

The maximal isometric tension per unit CSA at pH 6.6, ($P_{6.6}$) was elicited by a pCa 5.18 and a pCa 5.60 solution for hyper- and hypothyroid soleus muscle fibres respectively (Fig. 6.2). $P_{6.6}$ was 9% higher in hyper- than in hypothyroid soleus fibres (Table 6.4).

In the presence of high [Ca^{2+}] a decrease in the maximum tension was obtained, for the T/pCa relationships at pH 6.6. Thus at the end of the pH 6.6 T/pCa relationship, the tension per unit CSA produced by a pCa 4.10, pH 6.6 ($P_{Ca\ 6.6}$) (i.e. at maximal calcium activation at pH 6.6) solution was reduced in both hyper- and hypothyroid soleus fibres. With the hyperthyroid fibres having a significantly higher (37%) $P_{Ca\ 6.6}$ value than the hypothyroid fibres.

The isometric tension per unit CSA at pCa 4.14, pH 7.0 ($P_{7.0}$) (obtained in the same fibre, after measuring $P_{Ca\ 6.6}$) was slightly (7%) higher in hyper- than hypothyroid soleus fibres.

Table 6.4: Isometric tension and isometric tension per CSA of the soleus muscle fibres from hyper- and hypothyroid rats at pH 6.6 and pH 7.0.

		Hyperthyroid	Hypothyroid
Maximal tension at pH 6.6	Tension (mg)	32.11 ± 2.34 (28)	28.54 ± 1.98 (24)
	$P_{6.6}$ (kN/m^2)	118.69 ± 8.08	108.88 ± 7.21
Tension at pCa 4.10, pH 6.6	Tension (mg)	21.38 ± 1.70 (28) *	15.46 ± 1.19 (24)
	$P_{Ca\ 6.6}$ (kN/m^2)	79.13 ± 6.17 *	57.57 ± 3.25
Tension at pCa 4.14, pH 7.0	Tension (mg)	48.04 ± 3.30 (24)	44.07 ± 3.04 (22)
	$P_{7.0}$ (kN/m^2)	180.80 ± 12.80	168.68 ± 9.32

* $P < 0.05$, hyper- vs. hypothyroid

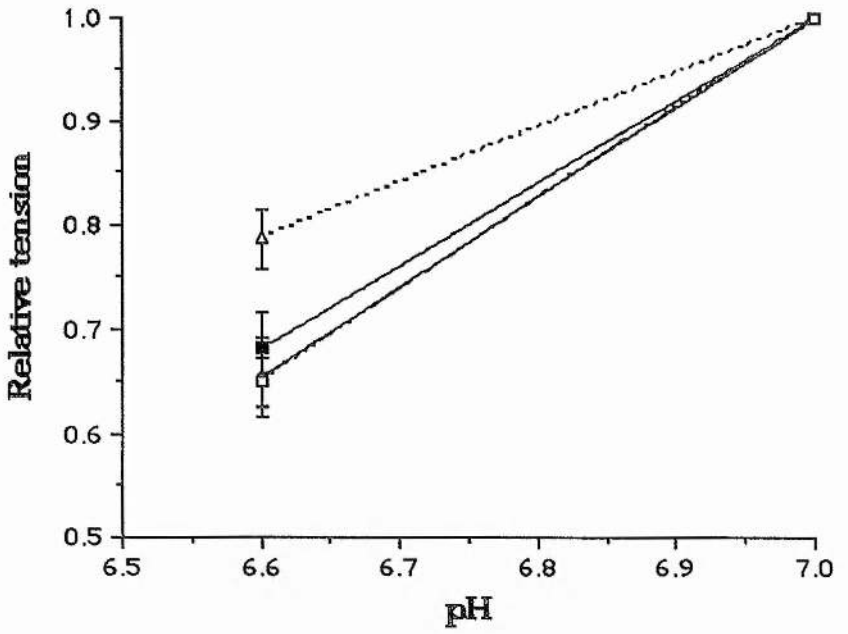
$P_{6.6}$ was elicited by a pCa 5.18 solution in both hyper- and hypothyroid TFL fibres (Fig. 6.3). At the end of the pH 6.6 T/pCa relationship, $P_{Ca\ 6.6}$ was reduced in both hyper- and hypothyroid TFL fibres. Although $P_{Ca\ 6.6}$ was 14% higher and $P_{7.0}$ was 25% higher in hyper- than hypothyroid TFL fibres, both were non significant (Table 6.5).

Table 6.5: Isometric tension and isometric tension per CSA of TFL muscle fibres from hyper- and hypothyroid rats at pH 6.6 and pH 7.0.

		Hyperthyroid	Hypothyroid
Maximal tension at pH 6.6	Tension (mg)	35.90 ± 4.88 (11)	35.15 ± 3.76 (20)
	$P_{6.6}$ (kN/m^2)	91.09 ± 8.84	97.31 ± 10.15
Tension at pCa 4.10, pH 6.6	Tension (mg)	35.30 ± 5.04 (11)	27.96 ± 2.89 (20)
	$P_{Ca\ 6.6}$ (kN/m^2)	89.44 ± 8.71	78.24 ± 8.31
Tension at pCa 4.14, pH 7.0	Tension (mg)	57.41 ± 7.65 (10)	42.68 ± 4.22 (19)
	$P_{7.0}$ (kN/m^2)	146.52 ± 12.78	117.39 ± 11.74

Expressing $P_{6.6}$ relative to $P_{7.0}$ (Fig. 6.4) shows that the depression of tension due to a reduction in pH from 7.0 to 6.6 occurs to a similar extent in hyper- soleus, hypo- soleus and hyperthyroid TFL fibres whilst the hypothyroid TFL fibres, although depressed, maintain a significantly larger proportion of the tension obtained at pH 7.0.

Fig. 6.4: Variations in maximum tension with a change in pH in hyperthyroid soleus (—■—), hypothyroid soleus (·····□·····), hyperthyroid TFL (—▲—) and hypothyroid TFL (·····△·····) single skinned fibres. Relative tensions were obtained by dividing the maximum isometric tension at pH 6.6 ($P_{6.6}$) by the maximum isometric tension in that same fibre at pH 7.0 ($P_{7.0}$). Each point represents the mean of values from 10 to 24 fibres. Hypo- TFL > hyper- soleus, $P < 0.05$; > hypo- soleus, $P < 0.005$; > hyper- TFL, $P < 0.05$.



The maximum tension at pH 6.6 ($P_{6.6}$) compared to the maximum tension at pH 7.0 ($P_{7.0}$) was found to decrease by 21 – 36% in fibres from both types of muscles, irrespective of thyroid status. This result is in qualitative agreement to those reported previously in skinned fibres from skeletal muscles from euthyroid animals where acidic pH was found to reduce isometric tension (Fabiato & Fabiato, 1978; Donaldson & Hermansen, 1978; Robertson & Kerrick, 1979; Donaldson, 1984; Chase & Kushmerick, 1988; Cooke et al., 1988; Metzger & Moss, 1987, 1988, 1990a; Godt & Nosek, 1989). Although, it has been shown in fibres from euthyroid animals that maximum tension is depressed more in fast than slow fibres, when pH is reduced to 6.5 (Donaldson, 1984; Metzger & Moss, 1987, 1990a), this is not in agreement with the present study. It is

possible that due to the milder acidity (pH 6.6) used in the present study this did not allow for differentiation between the four different groups, and in fact Metzger & Moss (1987, 1990a) found the differentiation between the slow and fast fibres to increase as pH was lowered further to pH 6.2, with respect to the depression of tension. The hypothyroid TFL fibres were the only group which was significantly different from the other three groups. As to why the hypothyroid TFL fibres had a significantly higher resistance to the fall in tension than the others, this could be due to the lower $P_{7.0}$ value obtained (Table 6.5) or due to hypothyroidism affecting the contractile apparatus, however the former of these explanations is the most likely.

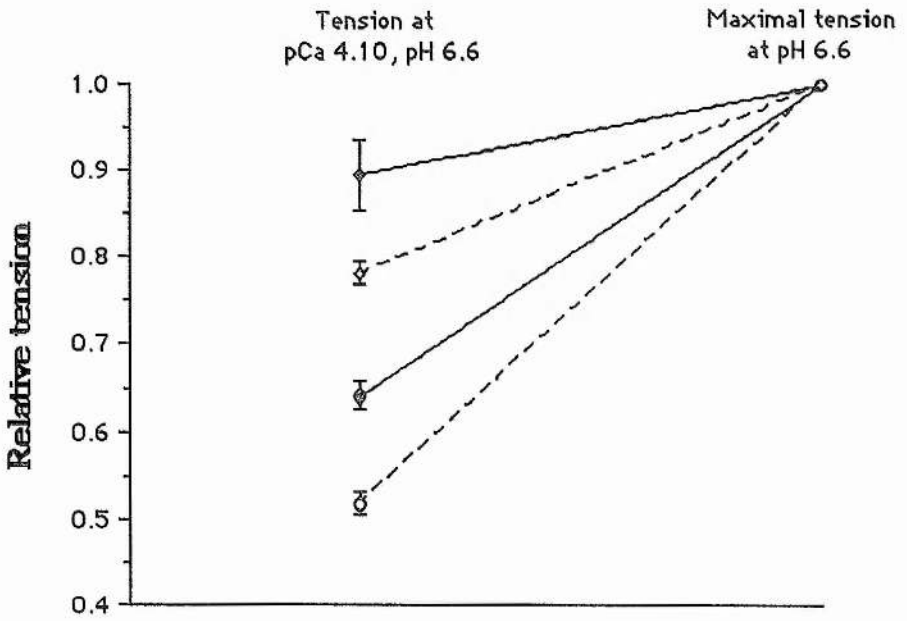
The mechanism by which low pH reduces tension is not known for certain. There is disagreement as to whether H^+ ions decrease (Blanchard et al. 1984) or leave unchanged (Fuchs, 1979) the affinity of Ca^{2+} binding sites on troponin. However, the finding that Ca^{2+} insensitive tension is similarly affected as Ca^{2+} activated tension at low pH (Metzger & Moss, 1988) makes the above disagreement irrelevant.

The decrease in tension at pH 6.6 in the present study is probably due to altered kinetics of cross bridge function leading to a reduced number of active cross bridges and/or to reduced tension of the individual cross bridge (Fabiato & Fabiato, 1978; Metzger & Moss, 1988). As to which of these possibilities is predominant can only be conjectured at. It could be largely due to a decrease of tension per cross bridge. Indeed it has been shown on live euthyroid fibres that decrease in contractile function leads to reduced number of cross bridges but most significantly to reduced tension per cross bridge (Edman & Lou, 1990). Furthermore, Metzger & Moss (1990a) found a reduction in both the number and the intrinsic strength of the cross bridges in fast euthyroid fibres whilst only the intrinsic strength of the cross bridges were

decreased without an apparent decrease in the number of cross bridges in slow euthyroid fibres. This result has been proposed as one of the reasons for the differential effect of H^+ ions of maximal tension on slow and fast muscle fibres.

Interestingly, $P_{Ca\ 6.6}$ relative to $P_{6.6}$ was also reduced in fibres from both types of muscles, irrespective of thyroid status (Fig. 6.5). The magnitude of the effect was dependent upon the fibre type and thyroid status. For example, the reduction was the largest in the direction hypo- soleus > hyper- soleus > hypo- TFL > hyper- TFL.

Fig. 6.5: Variations in tension with increasing $[Ca^{2+}]$ at pH 6.6, in hyperthyroid soleus (—●—), hypothyroid soleus (- - -○- - -), hyperthyroid TFL (—◆—) and hypothyroid TFL (- - -◇- - -) single skinned fibres. Relative tensions were obtained by dividing the tension at pCa 4.10, pH 6.6, ($P_{Ca\ 6.6}$) by the maximum tension in that same fibre at pH 6.6 ($P_{6.6}$). Each point represents the mean (± SEM) of values from 10 to 24 fibres. Hyper- TFL > hypo- TFL, $P < 0.05$; > hyper- soleus, $P < 0.005$; > hypo- soleus, $P < 0.005$. Hypo- TFL > hyper- soleus, $P < 0.005$; > hypo- soleus, $P < 0.005$. Hyper- soleus > hypo- soleus, $P < 0.005$.



The decrease in tension from the maximum tension obtained at pH 6.6 as the $[Ca^{2+}]$ increased was 10 – 21% in TFL fibres and 35 – 48% in soleus fibres.

This decrease was a direct consequence of dysthyreosis, because where investigators have looked at the effects of pH on slow or fast euthyroid muscle fibres, no one has reported significant lowering of tension with increasing $[Ca^{2+}]$. However, a decrease in tension has been reported for cardiac cells (Fabiato & Fabiato, 1978; Godt & Nosek, 1989) with supra optimal $[Ca^{2+}]$. This was related to the decrease seen in myofibrillar ATPase activity (Portzehl et al., 1969) when $[Ca^{2+}]$ is increased above 10^{-4} M. Therefore, it is possible that dysthyreosis shifted the sensitivity of myofibrillar ATPase to H^+ ions in such a manner as to achieve maximal tension at about 10^{-5} M and any further increase in $[Ca^{2+}]$ shows a decrease in tension as seen. This would also be consistent with the observed decrease in V_{max}.

This decrease in tension is likely to be due to a decrease in the number of strong cross bridges. Since it has been suggested (Metzger & Moss, 1990a) that tension per cross bridge attachment at constant pH is unaffected by changes in $[Ca^{2+}]$.

In conclusion, the results obtained for the Ca^{2+} sensitivity, maximal tension and V_{max} are consistent with the idea that hypo- and hyperthyroidism merely altered the dynamic equilibrium of the regulatory and contractile proteins from slow to fast in the direction hypo- to hyperthyroid. Although, no differentiation, with respect to contractile dysfunction, of slow and fast fibres was observed. This is probably a reflection of the milder acidity used in the present study.

CONTRACTILE PROPERTIES OF HYPER-/HYPOTHYROID SOLEUS AND TFL FIBRES AT 25 °C, pH 7.0, 7.5 mM P_i

For both the soleus and TFL muscle fibre measurements, the weight and core temperature of the animals was slightly higher and lower respectively, in hyper- than in hypothyroid animals (Table 7.1). All the fibres sampled between the two different thyroid states, within each muscle type, had similar diameters. This suggests that (within each muscle type) the same fibre type was sampled between the two different thyroid states.

Table 7.1: Measurements of soleus and TFL muscle fibres from hyper- and hypothyroid rats at 7.5 mM P_i and 0 mM P_i. $V_{max\ 7.5mM}$ was measured at pCa 4.14, pH 7.0, 7.5 mM P_i, at the end of the 7.5 mM P_i T/pCa relationship. $V_{max\ 7.0}$ was measured at pCa 4.14, pH 7.0, after having obtained $V_{max\ 7.5mM}$ in the same fibre.

	Hyperthyroid	Hypothyroid
	SOLEUS	
Rat weight (g)	398.80 ± 7.82 (N = 4)	385.00 ± 5.65 (N = 4)
Core temperature (°C)	37.28 ± 0.19 (4)	36.25 ± 0.25 (4)
Fibre diameter (μm)	59.68 ± 1.51 (14)	61.02 ± 1.96 (16)
$V_{max\ 7.5mM}$ (L_0S^{-1})	4.72 ± 0.57 (12)	4.52 ± 0.52 (15)
$V_{max\ 7.0}$ (L_0S^{-1})	4.97 ± 0.58 (12)	4.63 ± 0.42 (15)
	TFL	
Rat weight (g)	404.50 ± 6.25 (N = 4)	374.33 ± 16.68 (N = 3)
Core temperature (°C)	37.00 ± 0.20 (4)	36.17 ± 0.44 (3)
Fibre diameter (μm)	68.49 ± 3.80 (15)	71.09 ± 3.95 (13)
$V_{max\ 7.5mM}$ (L_0S^{-1})	12.44 ± 1.49 (13)	11.56 ± 1.40 (12)
$V_{max\ 7.0}$ (L_0S^{-1})	12.88 ± 1.38 (12)	11.66 ± 1.24 (11)

EFFECT OF P_i UPON V_{max}

Within both muscle types, the hyper- and hypothyroid $V_{max\ 7.0}$ values were only slightly (non significant) higher than $V_{max\ 7.5mM}$ values (Table 7.1). Therefore, in general, the V_{max} values were unchanged in both soleus and TFL fibres at increased $[P_i]$, irrespective of thyroid status, which agrees with results obtained by previous investigators from euthyroid fibres (Luney & Godt, 1987; Chase & Kushmerick, 1988; Cooke et al., 1988; Pate & Cooke, 1989a).

The experimentally observed results (above), agree with theoretical thermodynamic predictions, of V_{max} being unchanged at increased $[P_i]$. In this respect, Pate & Cooke (1989b) concluded that there is little effect on V_{max} because at physiologically relevant concentrations of P_i, the release of P_i occurs near the free energy minimum of the AM-ADP-P_i state to the highly strained AM'-ADP state (Fig. 1.1, step 5). Although increased P_i reverses this step, myosin heads near the minimum of the AM-ADP-P_i state generate little tension, and they release P_i before they generate significant negative work.

EFFECT OF P_i UPON Ca^{2+} SENSITIVITY

Generally, for any given calcium concentration, fibres from both soleus and TFL muscles, generated significantly higher tensions at 0 mM P_i than at 7.5 mM P_i, irrespective of thyroid status (Figs. 7.1 & 7.2).

Generally, in both thyroid states, irrespective of muscle type, when the tensions obtained at each phosphate concentration, were scaled relative to the maximum tension obtained (in the same fibre) at the same phosphate concentration or scaled relative to the tension obtained (in the same fibre) at pCa 4.14, 0 mM P_i, the relative T/pCa relationships were shifted to the right at 7.5 mM P_i with respect to those T/pCa relationships obtained at 0 mM P_i. This indicates a reduced calcium sensitivity of tension development with increased P_i, i.e. a higher free $[Ca^{2+}]$ was required to obtain the same tension when the inorganic phosphate concentration was increased.

The results above are similar to those results reported by investigators using euthyroid slow and fast fibres (Brandt et al., 1982; Hoar et al., 1987; Godt & Nosek, 1989; Millar & Homsher, 1990; Martyn & Gordon 1992). Although, at present there are no known reports of any differential effect of P_i on the Ca^{2+} sensitivity between slow and fast fibres or between the two types of fast fibres. It has been reported by Godt & Nosek (1989) that P_i affects the Ca^{2+} sensitivity of cardiac fibres more than fast fibres. This tends to agree with the present results which showed a larger decrease in calcium sensitivity in the soleus fibres than the TFL fibres irrespective of the thyroid status.

Fig. 7.1: Effect of P_i on single skinned fibres from hyperthyroid and hypothyroid soleus muscles. Mean values (± SEM) at 0 mM P_i, (——■——) and at 7.5 mM P_i, (·····□·····) for hyperthyroid fibres and at 0 mM P_i, (——●——) and at 7.5 mM P_i, (·····○·····) for hypothyroid fibres were obtained from 12 to 28 fibres. In all the fibres studied at 7.5 mM P_i, the maximum tension was always achieved at a free [Ca^{2+}] well below pCa 4.14, and higher Ca^{2+} led to significantly lower tension. Thus data for these fibres is scaled relative to the actual maximum tension obtained.

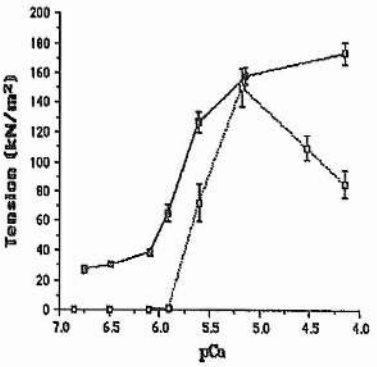
a) & e) Isometric T/pCa relationships.

b) & f) Relative T/pCa relationships. For the 7.5 mM P_i T/pCa curve, the isometric tension at each [Ca^{2+}] at 7.5 mM P_i, was scaled relative to the maximum tension generated in the same fibre at 7.5 mM P_i ($P_{7.5mM}$).

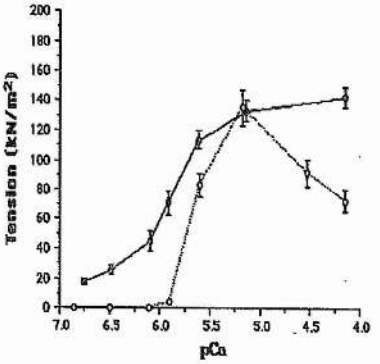
c) & g) Relative T/pCa relationships. For the 7.5 mM P_i T/pCa curve, the isometric tension at each [Ca^{2+}] at 7.5 mM P_i, was scaled relative to the maximum tension generated in the same fibre at 0 mM P_i ($P_{7.0}$).

d) & h) Average Hill plots of individual relative ($P_{7.5mM}$) T/pCa relationships.

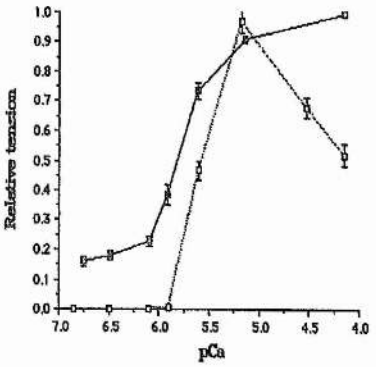
a) hyperthyroid Soleus



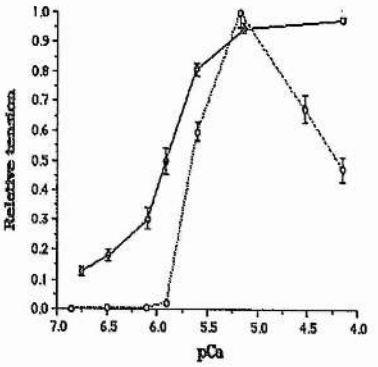
e) hypothyroid Soleus



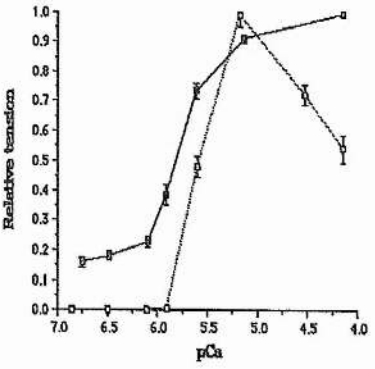
b) hyperthyroid Soleus



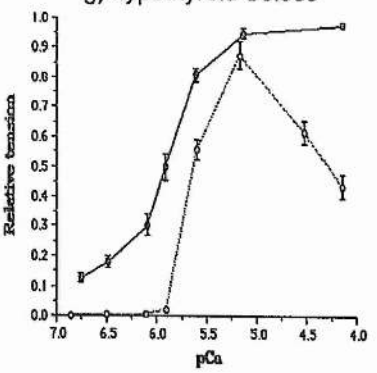
f) hypothyroid Soleus



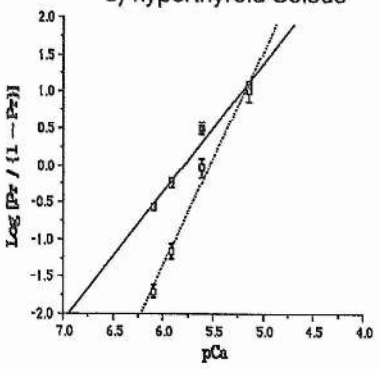
c) hyperthyroid Soleus



g) hypothyroid Soleus



d) hyperthyroid Soleus



h) hypothyroid Soleus

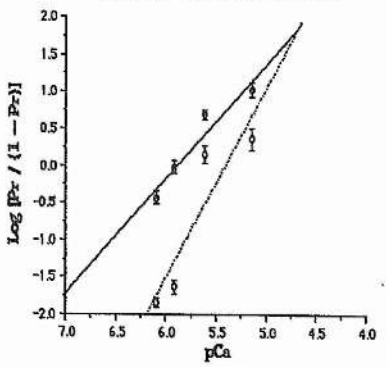


Fig. 7.2: Effect of P_i on single skinned fibres from hyperthyroid and hypothyroid soleus muscles. Mean values (± SEM) at 0 mM P_i, (——▲——) and at 7.5 mM P_i, (·····△·····) for hyperthyroid fibres and at 0 mM P_i, (——◇——) and at 7.5 mM P_i, (·····◇·····) for hypothyroid fibres were obtained from 12 to 18 fibres. In all the fibres studied at 7.5 mM P_i, the maximum tension was always achieved at a free $[Ca^{2+}]$ well below pCa 4.14, and higher Ca^{2+} led to significantly lower tension. Thus data for these fibres is scaled relative to the actual maximum tension obtained.

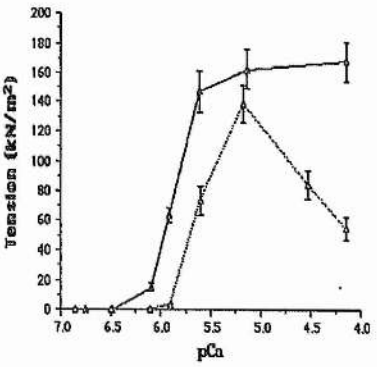
a) & e) Isometric T/pCa relationships.

b) & f) Relative T/pCa relationships. For the 7.5 mM P_i T/pCa curve, the isometric tension at each $[Ca^{2+}]$ at 7.5 mM P_i, was scaled relative to the maximum tension generated in the same fibre at 7.5 mM P_i ($P_{7.5mM}$).

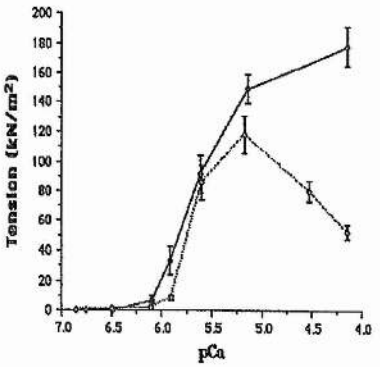
c) & g) Relative T/pCa relationships. For the 7.5 mM P_i T/pCa curve, the isometric tension at each $[Ca^{2+}]$ at 7.5 mM P_i, was scaled relative to the maximum tension generated in the same fibre at 0 mM P_i ($P_{7.0}$).

d) & h) Average Hill plots of individual relative ($P_{7.5mM}$) T/pCa relationships.

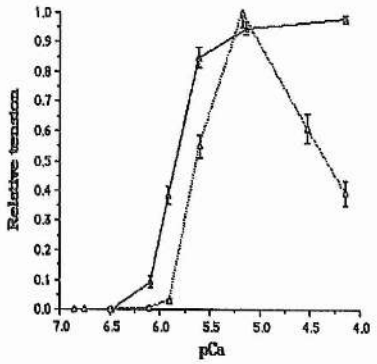
a) hyperthyroid TFL



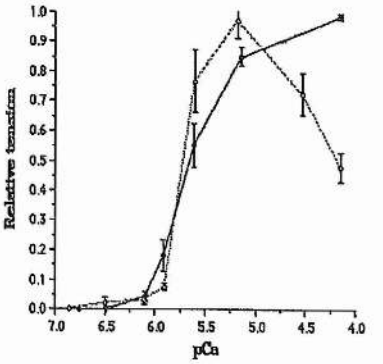
e) hypothyroid TFL



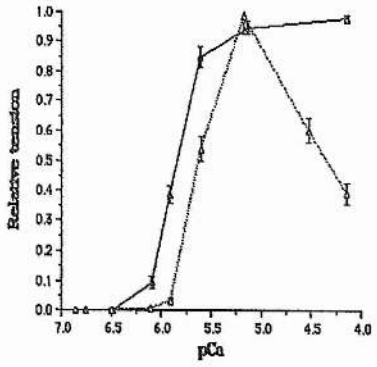
b) hyperthyroid TFL



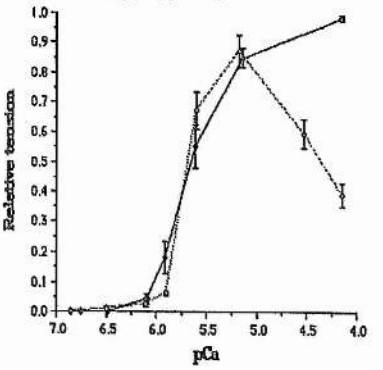
f) hypothyroid TFL



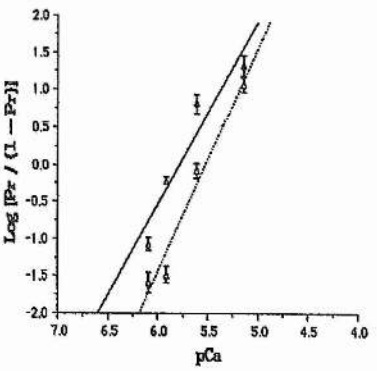
c) hyperthyroid TFL



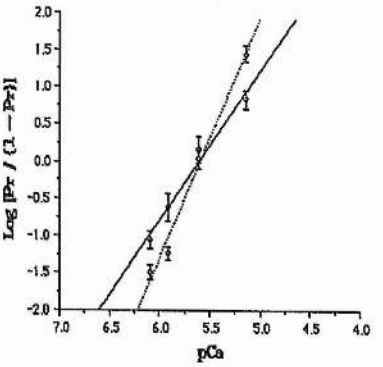
g) hypothyroid TFL



d) hyperthyroid TFL



h) hypothyroid TFL



The Hill plots (Figs. 7.1 & 7.2) show that when P_i was increased from 0 mM to 7.5 mM, the free [Ca^{2+}] required to obtain pCa_{TH} increased by a factor of 7.59 in hypothyroid soleus fibres as compared to a factor of 4.17 in hyperthyroid soleus fibres, and by a factor of 2.40 in hyperthyroid TFL fibres as compared to a factor of 2.00 in hypothyroid TFL fibres (Tables 7.2 & 7.3).

Table 7.2: Summary of Hill data of single skinned soleus fibres from hyper- and hypothyroid rats which have been analysed (in different fibre preparations) in various Ca^{2+} activating solutions of 7.5 mM P_i and 0 mM P_i.

	Hyperthyroid		Hypothyroid	
	0 mM P_i	7.5 mM P_i	0 mM P_i	7.5 mM P_i
pCa_{TH}	6.67	6.05	6.87	5.99
pCa_{50}	5.80	5.52	5.89	5.40
n	1.73 ± 0.10 $^+$	2.87 ± 0.13	1.54 ± 0.15 *	2.54 ± 0.41
ratio of n	1.66		1.65	

* $P < 0.05$, 0 mM vs. 7.5 mM P_i (hypo-); $^+$ $P < 0.005$, 0 mM vs. 7.5 mM P_i (hyperthyroid)

Table 7.3: Summary of Hill data of single skinned fibres from hyper- and hypothyroid TFL muscles which have been analysed (in different fibre preparations) in various Ca^{2+} activating solutions of 0 mM P_i and 7.5 mM P_i.

	Hyperthyroid		Hypothyroid	
	0 mM P_i	7.5 mM P_i	0 mM P_i	7.5 mM P_i
pCa_{TH}	6.39	6.01	6.35	6.05
pCa_{50}	5.77	5.51	5.60	5.59
n	2.44 ± 0.28	2.99 ± 0.19	1.99 ± 0.10 $^+$	3.22 ± 0.13
ratio of n	1.23		1.62	

$^+$ $P < 0.005$, 0 mM vs. 7.5 mM P_i (hypothyroid)

The free [Ca^{2+}] required to obtain pCa_{50} increased by a factor of 3.09 in hypothyroid soleus fibres as compared to a factor of 1.91 in hyperthyroid soleus fibres, and by a factor of 1.82 in hyperthyroid TFL fibres as compared to a factor of 1.02 in hypothyroid TFL fibres.

Thus, in all cases the sensitivity of the contractile apparatus decreased when phosphate was increased to 7.5 mM but the decrease in sensitivity was larger in hypo- than in hyperthyroid soleus fibres whilst it was larger in hyper- than in hypothyroid TFL fibres.

The Hill coefficient, n increased to a similar extent in both hyper- and hypothyroid soleus fibres whereas, n increased by 1.23-fold in hyperthyroid TFL fibres as compared to a 1.62-fold increase in hypothyroid fibres. This increase in the Hill coefficient, n, in the soleus and TFL fibres irrespective of thyroid status was also observed in euthyroid slow and fast fibres (Brandt et al., 1982; Hoar et al., 1987; Millar & Homsher, 1990).

A possible explanation for the decrease in calcium sensitivity may be that, P_i might affect Ca^{2+} sensitivity by direct effects on filament charge. For example, P_i could affect calcium binding if elevated P_i caused a change in myofilament charge density. However, because the myofilaments have a net negative charge at pH 7.0 (Collins & Edwards, 1971) P_i binding to charged sites would increase myofilament charge density and lead to an increase in calcium sensitivity rather than the observed decrease (Martyn & Gordon, 1988)

Alternatively, if P_i decreases tension mainly by shifting the strongly bound cross bridges to the weakly bound cross bridges as is thought to happen (see next section) then this could partially explain the effect of P_i on calcium sensitivity of the contractile apparatus in as much as attached cross bridges are thought to increase the calcium sensitivity of Tn on the thin filament through co-operative interactions (Bremel &

Weber, 1972; Fuchs, 1977; Brozovich et al., 1988). In addition, it could also explain the observed increase in the Hill coefficient, n.

That is, as [P_i] increases, the number of strong cross bridges decreases; and so for a given [Ca^{2+}], the number of active thin filament units decreases, and so isometric tension and calcium sensitivity both decrease. With a low calcium sensitivity, thin filament activation will be largely limited by the binding of strong cross bridges which is highly co-operative (Trybus & Taylor, 1980; Greene & Eisenberg, 1980), so the Hill coefficient, n increases.

As outlined earlier many other factors which can influence the position and steepness of the T/pCa relationships are possible target sites for P_i to decrease Ca^{2+} sensitivity. For example P_i might alter Ca^{2+} binding. However, evidence suggests (Kentish & Palmer, 1989) that elevated P_i does not affect calcium binding to TnC.

Furthermore, if the decrease in Ca^{2+} sensitivity was due solely to a decrease in strong cross bridges then the effect of P_i to increase the Hill coefficient, n should have been differentiated in the same manner. However, this was not the case, and in fact, P_i increased the Hill coefficient, n to a similar extent in all four experimental states, suggesting that only part of the decrease in Ca^{2+} sensitivity was mediated through a decrease in the number of strong cross bridges and other factors, are able to reduce Ca^{2+} sensitivity without affecting the co-operativity.

In essence the rightward shift of the T/pCa relationship at increased inorganic phosphate concentration in the present study is consistent with the idea that hypo- and hyperthyroidism merely shifted the equilibrium of the fibres with respect to the regulatory and contractile proteins from slow to fast respectively. Moreover, the effect was larger in the soleus than TFL fibres.

EFFECT OF P_i UPON MAXIMUM TENSION

The maximal isometric tension per unit CSA at 7.5 mM P_i ($P_{7.5mM}$) was elicited by a pCa 5.14 solution for both hyper- and hypothyroid soleus fibres (Fig. 7.1). $P_{7.5mM}$ was 11% (non-significant) higher in hyper- than hypothyroid fibres (Table 7.4).

In the presence of high [Ca^{2+}] a decrease in the maximum tension was obtained for the T/pCa relationships at 7.5 mM P_i. Thus at the end of the 7.5 mM P_i T/pCa relationship, the isometric tension per unit CSA produced by a pCa 4.14, 7.5 mM P_i ($P_{Ca\ 7.5mM}$) (i.e. at maximal calcium activation at 7.5 mM P_i) solution was higher (18%) in hyper- than in hypothyroid soleus fibres.

The isometric tension per unit CSA at pCa 4.14, 0 mM P_i ($P_{7.0}$) (obtained in the same fibre, after measuring $P_{Ca\ 7.5mM}$) was essentially unchanged between hyper- and hypothyroid soleus fibres.

Table 7.4: Isometric tension and isometric tension per CSA of soleus muscle fibres from hyper- and hypothyroid rats at various Ca^{2+} concentrations in solutions of 7.5 mM P_i and 0 mM P_i.

		Hyperthyroid	Hypothyroid
Maximal tension at 7.5 mM P_i	Tension (mg)	42.02 ± 6.52 (14)	40.33 ± 5.78 (16)
	$P_{7.5mM}$ (kN/m^2)	150.36 ± 12.76	135.13 ± 12.44
Tension at pCa 4.14, 7.5 mM P_i	Tension (mg)	23.81 ± 3.45 (14)	21.33 ± 2.91 (16)
	$P_{Ca\ 7.5mM}$ (kN/m^2)	85.23 ± 8.98	72.50 ± 7.56
Tension at pCa 4.14, 0 mM P_i	Tension (mg)	43.36 ± 6.78 (12)	45.40 ± 6.09 (15)
	$P_{7.0}$ (kN/m^2)	155.17 ± 13.49	153.96 ± 12.93

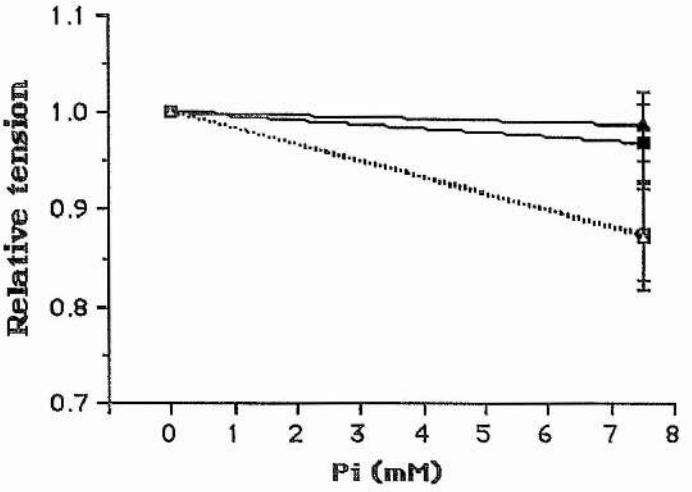
For TFL fibres $P_{7.5mM}$ was elicited by a pCa 5.14 solution in both hyper- and hypothyroid TFL fibres (Fig. 7.2). Although, $P_{7.5mM}$ was 17% higher in hyper- than hypothyroid fibres it was non-significant. At the end of the 7.5 mM P_i T/pCa relationship, $P_{Ca\ 7.5mM}$ was reduced to a similar extent in both hyper- and hypothyroid TFL fibres. $P_{7.0}$ was slightly higher in hyper- than hypothyroid TFL fibres (Table 7.5).

Table 7.5: Isometric tension and isometric tension per CSA of TFL muscle fibres from hyper- and hypothyroid rats at various Ca^{2+} concentrations at 7.5 mM P_i and 0 mM P_i.

		Hyperthyroid	Hypothyroid
Maximal tension at 7.5 mM P_i	Tension (mg)	49.70 ± 5.21 (15)	39.75 ± 6.51 (13)
	$P_{7.5mM}$ (kN/m^2)	138.36 ± 12.41	118.32 ± 12.57
Tension at pCa 4.14, 7.5 mM P_i	Tension (mg)	19.85 ± 3.15 (15)	18.25 ± 3.57 (13)
	$P_{Ca\ 7.5mM}$ (kN/m^2)	54.87 ± 7.65	52.73 ± 5.06
Tension at pCa 4.14, 0 mM P_i	Tension (mg)	49.29 ± 5.78 (13)	46.00 ± 5.76 (12)
	$P_{7.0}$ (kN/m^2)	137.38 ± 15.49	135.16 ± 11.20

Expressing $P_{7.5mM}$ relative to $P_{7.0}$ (Fig. 7.3) shows that the maximum tension at 7.5 mM P_i compared to the maximum tension at 0.0 mM P_i decreased by 1 – 12%, And only the hypothyroid fibres (from both muscle types) were significantly lower from control values.

Fig. 7.3: Reduction in maximum tension due to an increase of P_i concentration in hyperthyroid soleus (—■—), hypothyroid soleus (·····□·····), hyperthyroid TFL (—▲—) and hypothyroid TFL (·····△·····) single skinned fibres. Relative tensions were obtained by dividing the maximum isometric tension at 7.5mM P_i ($P_{7.5mM}$) by the maximum isometric tension in that same fibre at pH 7.0 ($P_{7.0}$). Each point represents the mean (± SEM) of values from 12 to 13 fibres.



This result is in qualitative agreement to those reported previously, in skinned fibres from slow and fast muscle fibres from euthyroid animals where increased phosphate concentration was found to reduce isometric tension, although these studies showed larger depressions in tension than the present study, this was primarily due to a larger P_i concentration used (Hibberd et al., 1985; Cooke & Pate, 1985; Kwai, 1986; Webb et al., 1986; Nosek et al., 1987; Hoar et. al., 1987; Kwai et al., 1987; Cooke et al., 1988; Pate & Cooke, 1989a; Godt & Nosek, 1989; Neil & Homsher, 1990; Martyn & Gordon, 1992). Where P_i was increased to 7.5mM little or no change was observed, concurring totally with the present study (Brandt et al., 1982).

It has been shown that P_i reduces maximal tension by shifting the strong tension producing cross bridges to the weak non tension producing cross bridges (Hibberd & Trentham, 1986; Webb et al., 1986; Dantzig et al., 1987; Cooke et al., 1988; Pate & Cooke, 1989a, b). In

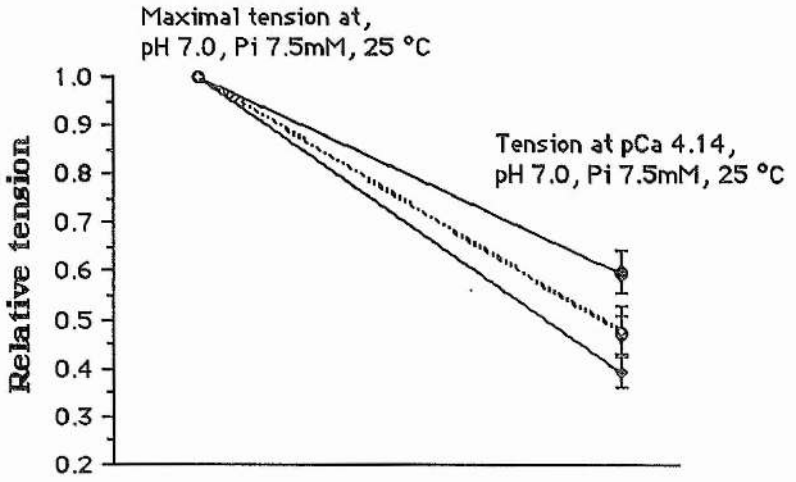
short, P_i reverses step 5 (Fig 1.1) of the cross bridge cycle (P_i release) by shifting the distribution of cross bridges towards those states with a full complement of bound products (ADP and P_i).

Therefore, since hypo- and hyperthyroidism is merely changing the dynamic equilibrium of the contractile proteins. The decrease in tension in the present study is mainly due to a decrease in the number of strong cross bridges as oppose to a decrease in the tension per cross bridge. However, a recent report by Martyn & Gordon (1992) found P_i to decrease tension but not stiffness (from low to 10mM P_i), at maximal Ca^{2+} activation, suggesting decrease in tension is due to a decrease in the tension per cross bridge.

Interestingly, $P_{Ca\ 7.5mM}$ relative to $P_{7.5mM}$ was also reduced in fibres from both types of muscles, irrespective of thyroid status (Fig. 7.4). The magnitude of this effect was 46 – 52% in slow fibres and 52 – 60% in TFL fibres. The reduction was largest in the direction hyper- TFL > hypo- TFL > hypo- soleus > hyper- soleus.

As outlined earlier, this reduction occurs in cardiac cells and could be due to the effect on the myofibrillar ATPase activity i.e., P_i decreases tension due to a decrease in myofibrillar ATPase activity. However V_{max} was unchanged, thus it is unlikely for myofibrillar ATPase activity to be reduced. therefore, another mechanism is implicated (see below).

Fig. 7.4: Reduction of tension at high [Ca^{2+}] (pCa 4.14) in hyperthyroid soleus (—●—), hypothyroid soleus (·····○·····), hyperthyroid TFL (—◆—) and hypothyroid TFL (·····◇·····) single skinned fibres. Relative tensions were obtained by dividing the tension at pCa 4.14, 7.5mM P_i, pH 7.0 ($P_{Ca\ 7.5mM}$) by the maximum tension in that same fibre at 0.0mM P_i, pH 7.0 ($P_{7.5mM}$). Each point represents the mean (± SEM) of values from 12 or 13 fibres. Hyper-soleus> hyper- TFL, $P < 0.05$.



This decrease in tension in the presence of H^+ ions or P_i seems to suggest a role for calcium in decreasing tension. This is probably to protect the fibre from damage when under conditions of fatigue. This effect of negative inhibition by high [Ca^{2+}] has probably been unmasked due to dysthyreosis.

A possible mechanism for this decrease could be for Ca^{2+} to decrease the number of strong cross bridges (which is common effect of P_i and H^+ ions). In this respect Ca^{2+} has been shown to affect the cross bridge cycle (Metzger & Moss, 1990b, Millar & Homsher, 1990; Martyn & Gordon, 1992). Moreover, the differentiation of the effect between the four experimental groups could be due to presence of different isoforms of myosin heavy and light chains.

EFFECT OF THYROID STATUS ON SOLEUS AND TFL (WHOLE) MUSCLES

MYOFIBRILLAR ATPase ACTIVITY

The weight of the hyperthyroid animals (Table 8.1) was slightly (9%) but significantly ($P < 0.05$) higher than the hypothyroid animals. The basal metabolic rate was lower (2%) in hypo- than hyperthyroid animals as reflected by the decreased core temperature of hypo- animals with respect to hyperthyroid animals, although it was non-significant.

Table 8.1: Animal and muscle weights of hyper- and hypothyroid rats.

	Hyperthyroid	Hypothyroid
Rat weight (g)	407.00 ± 11.51 (N = 6) *	373.17 ± 15.09 (N = 6)
Core temperature (°C)	37.00 ± 0.26	36.33 ± 0.17
Soleus weight (mg)	166.20 ± 6.30	157.80 ± 4.90
Soleus/body ratio (mg/g)	0.41 ± 0.02	0.42 ± 0.02
TFL weight (mg)	313.40 ± 14.30	303.90 ± 11.70
TFL/body ratio (mg/g)	0.77 ± 0.04	0.81 ± 0.05

* $P < 0.05$, hyper- vs. hypothyroid

The mean soleus or TFL muscle weights were not significantly affected and therefore there was no real effect on the muscle weight/body weight ratio between hyper- and hypothyroid animals. This result is consistent with results obtained by previous investigators (Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nicol & Maybee, 1982) who used similar regimes to produce hypo- or hyperthyroidism as in the present study. Thus, this result suggests that the growth and development of the animals was effectively normal, without any net anabolic or catabolic effects on either the rats as a whole or the individual muscles, despite

the differing thyroid hormone levels.

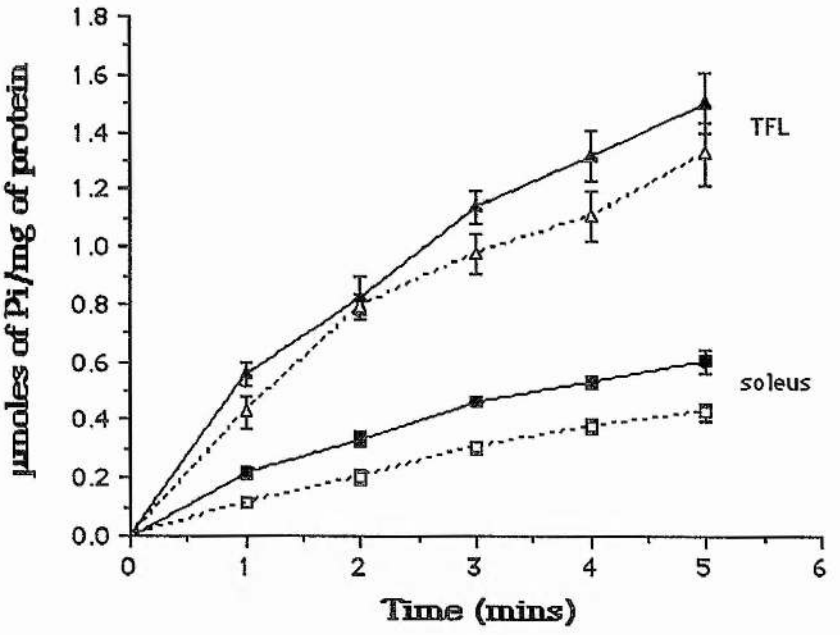
For the soleus muscles, the Mg^{2+}, EGTA myofibrillar ATPase activity (Table 8.2) was 100% higher ($P < 0.005$) in hyper- than hypothyroid animals, whereas for the TFL muscles it was 41% higher (significant at the one tailed t-test level, $P < 0.05$). Similarly, the Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity (Table 8.2, Fig. 8.1) of the soleus muscles was 81% higher ($P < 0.05$) in hyper- than hypothyroid animals, whereas for the TFL muscles it was 16% higher (non-significant) in hyper- than hypothyroid animals.

Table 8.2: Mg^{2+}, EGTA and Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity of soleus and TFL muscles from hyper- and hypothyroid animals. Activity units are μmoles of P_i/mg of protein/min and yield is expressed as mg of protein/g muscle. Values are mean ± SEM from between 5 and 6 animals.

	Hyperthyroid	Hypothyroid
	Soleus	
EGTA activity	0.076 ± 0.008 +	0.038 ± 0.010
Ca^{2+} activity	0.176 ± 0.016 *	0.097 ± 0.009
Ca^{2+} sensitivity (%)	58.20 ± 1.91	64.53 ± 5.80
yield	87.74 ± 3.27	78.83 ± 6.25
	TFL	
EGTA activity	0.097 ± 0.012	0.069 ± 0.006
Ca^{2+} activity	0.418 ± 0.037	0.359 ± 0.039
Ca^{2+} sensitivity (%)	76.09 ± 2.76	82.62 ± 4.58
yield	87.23 ± 3.31	85.82 ± 3.81

* $P < 0.05$, + $P < 0.005$, hyper- vs. hypothyroid

Fig. 8.1: Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity of hyper- (—■—) and hypothyroid soleus (·····□·····) muscles and of hyper- (——▲——) and hypothyroid TFL (·····△·····) muscles. The Ca^{2+} activated myofibrillar ATPase activity of the myofibrils was calculated from the slope of the initial straight line portion of the curves. Values are mean ± SEM (which are indicated by the vertical bars or the size of the symbols used) from between 5 and 6 animals.



The 2.3-3.7 fold higher myofibrillar ATPase activity in fast hyper- and hypothyroid muscles compared to slow hyper- and hypothyroid muscles observed in the present study agrees favourably with the study of Baldwin et al. (1977) who found a 2.3-3.5 fold increase in fast euthyroid muscles compared to slow euthyroid muscles.

The 81% increase in the (Mg^{2+} activated Ca^{2+} regulated) myofibrillar ATPase activity in hyper- compared to hypothyroid soleus muscles is in quantitative agreement with the result obtained by Nwoye et al. (1982) who found myofibrillar ATPase activity to be 82% higher. Furthermore, the result is also in quantitative agreement with the results of investigators (Ianuzzo et al., 1977, 1980 Johnston et al., 1980a) who measured Ca^{2+} activated myosin ATPase activity.

Although, the present result contradicts the results of Fitts et al. (1980) who found no change in Mg^{2+} activated actomyosin ATPase activity in hyper- compared to euthyroid soleus muscles. The results obtained by Fitts et al. (1980) could be a consequence of comparing hyper- with euthyroid muscles as opposed to hyper- with hypothyroid, where the results could become significant. On the other hand, a recent study, by Fitzsimons et al. (1990) found no difference in the myofibrillar ATPase activity between hyper- and hypothyroid soleus muscles. The reason for this discrepancy does not seem apparent.

In fast muscle, no previous results are known to exist for the TFL muscle, but results obtained on other fast muscles, are in general agreement with the results obtained for the TFL muscles in the present study. For example, a fast muscle such as the (white) gastrocnemius has shown either a non-significant increase (8%) in the myofibrillar ATPase activity in hyper- compared to hypothyroid muscles (Fitzsimons et al., 1990) or a non-significant decrease in both the myofibrillar ATPase activity (14%) and the Ca^{2+} activated myosin ATPase activity (9%) of hypothyroid muscles compared to euthyroid muscles (Leijendekker & Hardveld, 1987). Furthermore, Ianuzzo et al. (1980) found the Ca^{2+} activated myosin ATPase activity of the fast plantaris muscle, relatively unchanged in hyper- compared to hypothyroid muscles.

However, measurements of myofibrillar ATPase activity in the (red) gastrocnemius muscle have shown a 53% (significant) increase in the myofibrillar ATPase activity of hyper- compared to hypothyroid muscles (Fitzsimons et al., 1990). Moreover, measurements of Ca^{2+} activated myosin ATPase activity in the fast EDL muscle have shown a 34% (significant) increase in the Ca^{2+} activated myosin ATPase activity of hyper- compared to hypothyroid muscles (Nwoye & Mommaerts, 1981).

Although, these results contradict the ones obtained for the TFL muscles in the present study and previous studies (Ianuzzo et al., 1980; Leijendekker & Hardveld, 1987; Fitzsimons et al., 1990) they can be explained as follows:

In the red gastrocnemius and the EDL muscles the majority fibre type is FOG whereas in the TFL, plantaris and white gastrocnemius muscles the majority fibre type is FG (Ariano et al., 1973; Armstrong & Phelps, 1984). Therefore, the difference in sensitivity of thyroid levels to the ATPase reaction seems to lie in the fibre types, and to this extent muscles composed primarily of FG fibres are less likely to be affected than muscles composed of FOG fibres because investigators (e.g., Nwoye & Mommaerts, 1981) have found fast muscle to be less responsive to thyroid hormone levels than slow muscle, and similarly it has been reported that within fast muscles, the fast muscles composed primarily of FG fibres are even less responsive than fast muscles composed primarily of FOG fibres (Sickles et al., 1987; Fitzsimons et al., 1990) and hence the apparent contradiction in the results.

The Ca^{2+} sensitivity between hyper- and hypothyroid muscles of the soleus or the TFL was unchanged. This concurs with the results obtained by Leijendekker & Hardveld (1987) who found no significant effect on the Ca^{2+} sensitivity of myofibrillar ATPase activity between hypo- and euthyroid muscles. The unchanged Ca^{2+} sensitivity between hyper- and hypothyroid muscles of the soleus or the TFL muscles tends to suggest that changes in the ATPase activity were due to the contractile proteins alone, as there was no significant loss of calcium sensitivity. Furthermore, since actin is highly conserved (Vanderckhove & Weber, 1979) then the ATPase activity changes are specifically due to changes in the myosin molecule and consequently due to the isoforms of myosin

heavy and light chains.

The yield of myofibrillar protein/g of muscle was not significantly different between hyper- and hypothyroid soleus or TFL muscles, and there was also no significant difference between the soleus and TFL muscles, irrespective of thyroid status (i.e. no significant difference between the four groups studied). This agrees with results obtained by previous investigators (Ianuzzo et al., 1977, 1980; Fitts et al., 1980) who found no change in the actomyosin or myosin yield either from slow or fast hypo- and hyperthyroid muscles.

This finding reinforces the above conclusion of no significant change in the growth and development of the animals. Secondly it suggests that mild dysthyreosis does not change the contractile material in either the soleus or TFL muscles to any significant effect. Thirdly it concurs with results of previous investigators (e.g., Close, 1972) who have shown no significant difference in the contractile material in slow and fast euthyroid muscles.

FIBRE POPULATIONS

The percentage (%) of FOG fibres was twice as high ($P < 0.05$) in hyper- than in hypothyroid soleus muscles and consequently the percentage of SO fibres was four-fold lower (Table 8.3, plates 8.1 & 8.2). This is because it has been shown that dysthyreosis has no effect on the total number of fibres in slow or fast muscles (Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nicol & Maybee, 1982) and therefore any change in fibre populations is due to inter conversion of fibre types.

Table 8.3: Percentage of various fibre types identified histochemically from soleus muscles of hyper- and hypothyroid animals. Values are mean ± SEM of between 4 and 6 animals.

Fibre type	Hyperthyroid	Hypothyroid
SO	67.04 ± 5.41 *	89.03 ± 2.60
FOG	32.96 ± 4.54 *	10.98 ± 2.60
IC	11.87 ± 3.99 *	1.65 ± 0.78
IIC	7.35 ± 1.73	7.33 ± 2.12

* $P < 0.05$, hyper- vs. hypothyroid

The higher percentage of FOG fibres in hyper- and the lower percentage of FOG fibres in hypothyroid soleus muscles and consequently decreased and increased levels of SO fibres respectively, observed in the present histochemical study confirm previous findings using a similar regime to obtain hypo- and hyperthyroidism as in the present study (Nicol & Bruce, 1981; Nicol & Johnston, 1981; Nicol & Maybee, 1982).

As can be seen in plates 8.1 and 8.2, in the soleus muscles there was a small number of fibres with SDH and myosin ATPase activities higher than SO fibres but less than FOG fibres. For the purpose of fibre

counting and calculating the percentage of SO and FOG fibres, these intermediate fibres were divided into two groups, those with a degree of staining approximately closest to that shown by FOG fibres (type IIC) and those whose degree of staining made them more like SO fibres (type IC). These transitional fibres corresponded to those described by Staron & Pette, 1987a, who found that the IC type fibres were histochemically more like type I (SO) fibres, whereas the IIC type fibres appeared histochemically to be more similar to IIA (FOG) fibres.

The percentage of type IC fibres was much higher in hyper- ($P < 0.05$) than hypothyroid soleus muscles whereas there was no apparent difference between the percentage of IIC fibres in the two different thyroid states.

The higher percentage of transitional fibres (IC and IIC) found in hyper- compared to hypothyroid soleus muscles is in total accord with the study by Muntener et al. (1987) who found a similar increase in the transitional fibres. The higher percentage of transitional fibres in hyperthyroid muscles, probably reflects the greater change which can be induced in the direction euthyroid to hyperthyroid (i.e., the potential increase in the number of fast fibres) as opposed to euthyroid to hypothyroid (i.e., the potential decrease in the number of slow fibres) soleus muscles.

Plate 8.1: Photographs of serial sections cut from hyperthyroid soleus muscles. Scale bar represents 50 μm.

a) SDH stain

b) Acid pre-incubated ATPase stain

c) ATPase stain (neutral fixation)

SO (Slow Oxidative) fibres stain moderately, intensely and lightly, whereas FOG (Fast Oxidative Glycolytic) fibres stain intensely, lightly and intensely for the SDH, acid pre-incubated and neutrally fixed stains respectively.

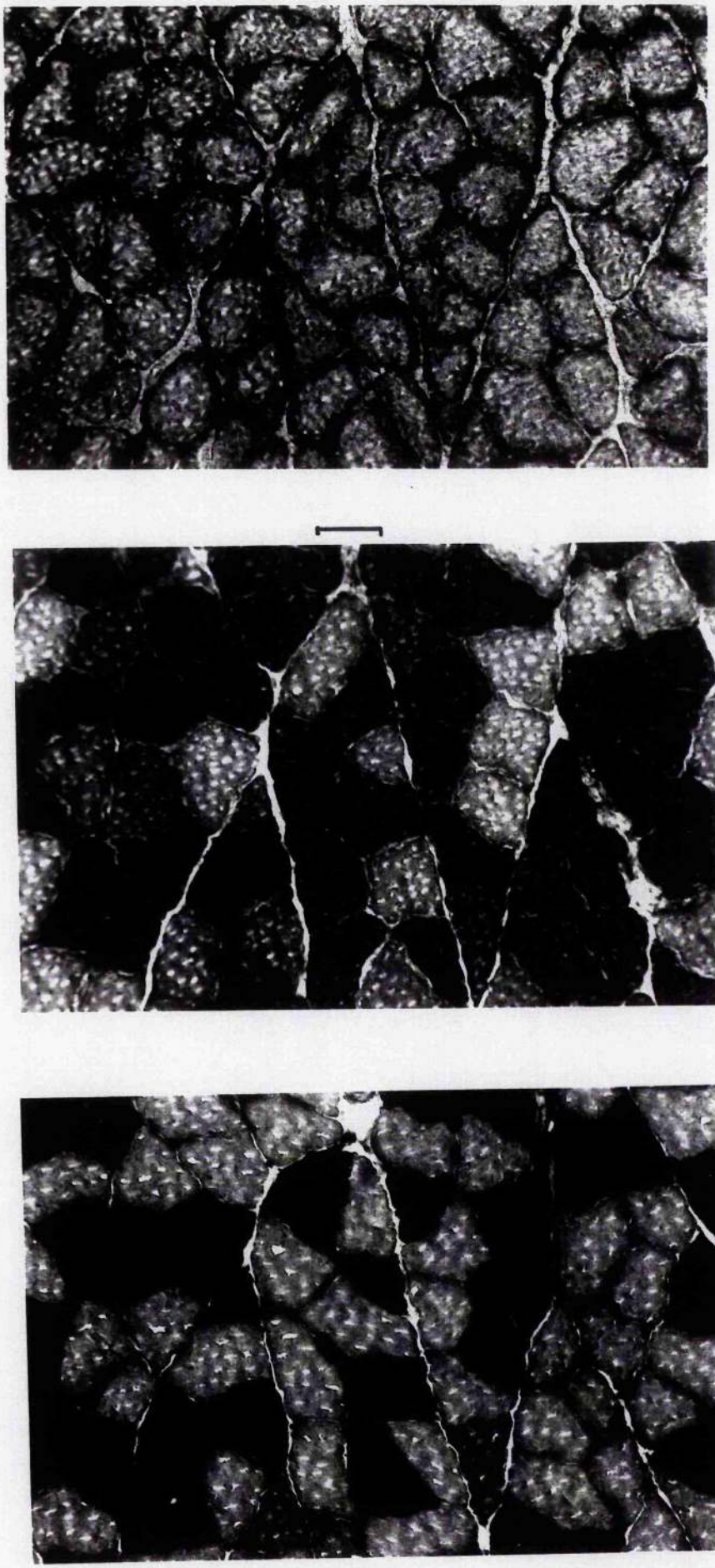


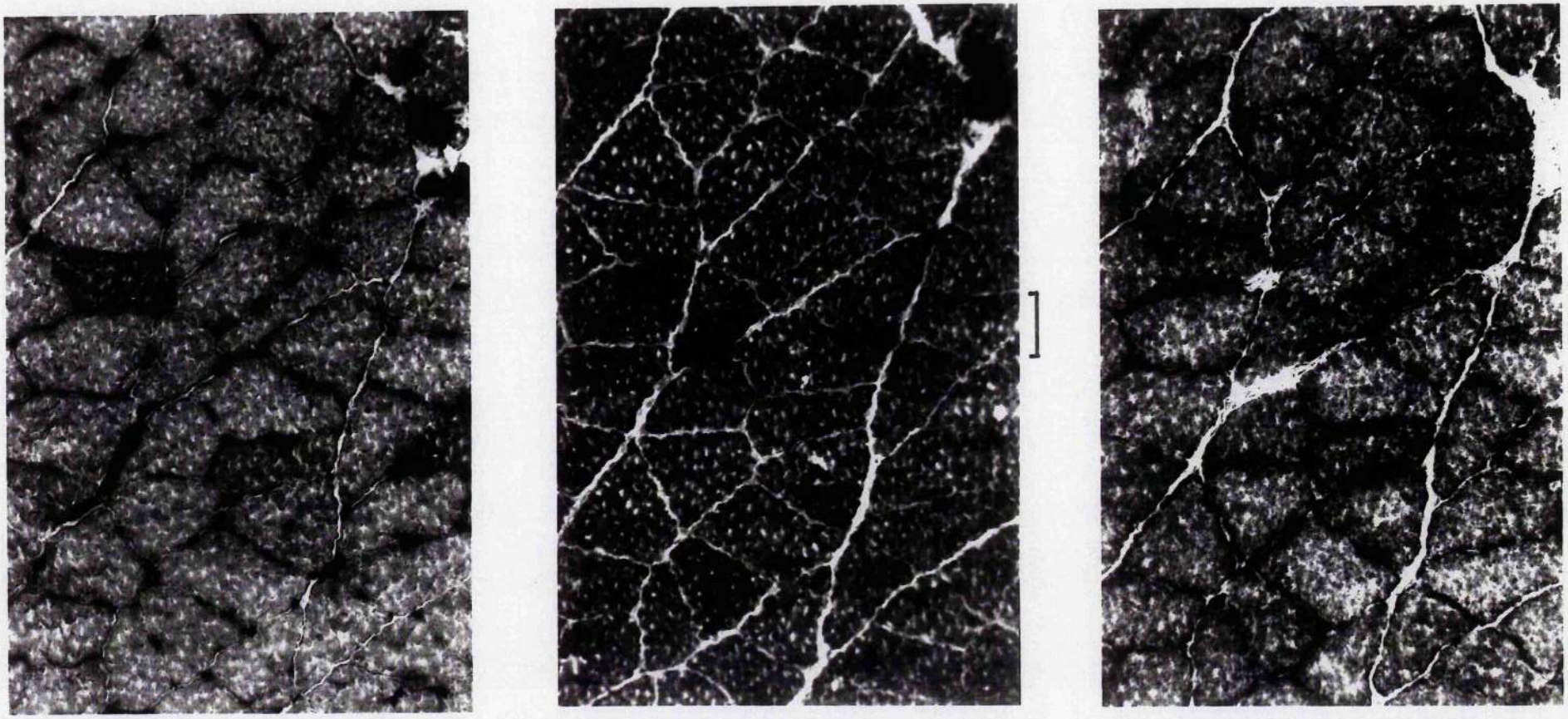
Plate 8.2: Photographs of serial sections cut from hypothyroid soleus muscles. Scale bar represents 50 μm.

a) SDH stain

b) Acid pre-incubated ATPase stain

c) ATPase stain (neutral fixation)

SO (Slow Oxidative) fibres stain moderately, intensely and lightly, whereas FOG (Fast Oxidative Glycolytic) fibres stain intensely, lightly and intensely for the SDH, acid pre-incubated and neutrally fixed stains respectively.



In the TFL muscles (Table 8.4, plate 8.3), the percentage of FG fibres was slightly higher in hyper- than hypothyroid muscles and consequently the percentage of FOG fibres was lower in the latter. However, although the percentage of FG fibres tended to be (significant at the one tailed t-test level) higher in hyper- than hypothyroid muscles, the results were not significant at the two tailed t-test level.

Table 8.4: Percentage of various fibre types identified histochemically from TFL muscles of hyper- and hypothyroid animals. Values are mean ± SEM of 5 animals, in both instances.

Fibre type	Hyperthyroid	Hypothyroid
FG	85.89 ± 1.62	79.77 ± 2.31
FOG	13.99 ± 1.69	20.23 ± 2.31

For the TFL muscles, the higher percentage of FG fibres in hyperthyroid muscles compared to hypothyroid muscles seem to contradict the results obtained by Sickles et al. (1987), who found no change when comparing hyper- with euthyroid muscles. However, this is more likely a consequence of comparisons being made between hyper- and euthyroid muscles as opposed to comparing hyper- with hypothyroid muscles as in the present study.

Moreover, Izumo et al. (1986) found no expression of the type IIA (FOG) gene in eu- or hyperthyroid TFL muscles, whereas the expression of this gene was newly induced in hypothyroid TFL muscles. Thus providing a possible explanation for the higher percentage of FOG fibres in the hypothyroid TFL muscle as observed.

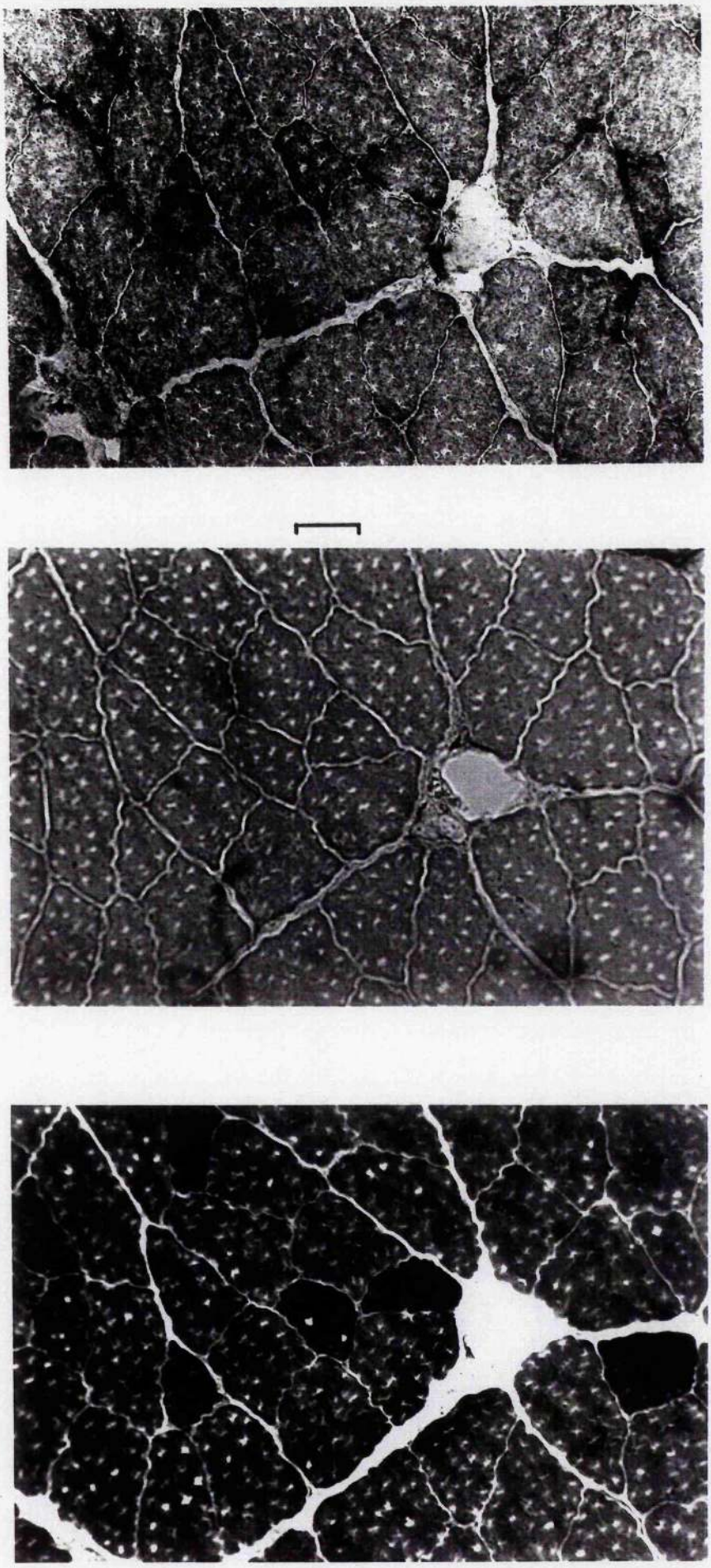
Plate 8.3: Photographs of serial sections cut from hypothyroid TFL muscles. Scale bar represents 50 μm.

a) SDH stain

b) Acid pre-incubated ATPase stain

c) ATPase stain (neutral fixation)

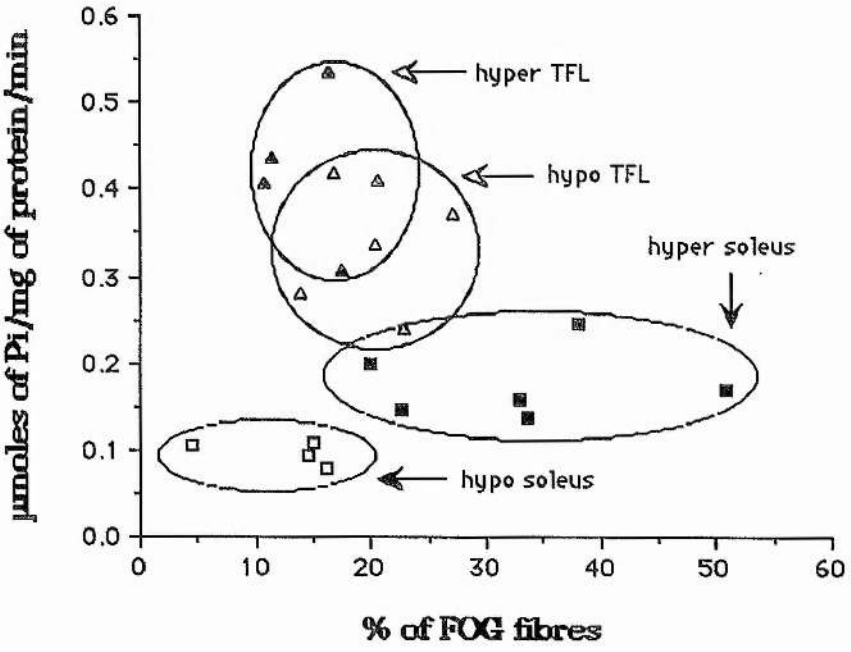
FG (Fast Glycolytic) fibres stain lightly, lightly and moderately, whereas FOG (Fast Oxidative Glycolytic) fibres stain intensely, lightly and intensely for the SDH, acid pre-incubated and neutrally fixed stains respectively.



COMPARISON OF ATPase ACTIVITY WITH FIBRE POPULATIONS

Plotting the (Mg^{2+} activated Ca^{2+} regulated) myofibrillar ATPase activity against the percentage of FOG fibres in a muscle reveals (Fig. 8.2), that for the soleus, there are two distinct groups representing the hyper- and hypothyroid muscles, i.e., the myofibrillar ATPase activity is higher in muscles from the hyperthyroid group which also contain a larger percentage of FOG fibres, whereas the myofibrillar ATPase activity is lower in muscles from the hypothyroid group, which contain a smaller percentage of FOG fibres. However, within each group, there doesn't seem to be a strong correlation of myofibrillar ATPase activity with the percentage of FOG fibres. And there isn't a strong correlation of these two parameters even when the two groups are combined.

Fig. 8.2: Plot of Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity vs. percentage of FOG fibres for hyper- (■) and hypothyroid soleus (□) muscles and for hyper- (▲) and hypothyroid TFL (△) muscles.



For TFL muscles, although there are two distinct groups, the overlap between them is much greater than the two soleus muscle groups. This is consistent with the evidence that slow muscles are more sensitive to thyroid hormone levels than fast muscles (e.g., Nwoyè & Mommaerts, 1981).

The myofibrillar ATPase activity is higher in TFL muscles from the hyperthyroid group, which contain a lower percentage of FOG fibres but a higher percentage of FG fibres (muscles containing primarily FG fibres have been shown to be have higher myofibrillar ATPase activity than muscles containing primarily FOG fibres e.g., Fitzsimons et al., 1990), whereas the myofibrillar ATPase activity is lower in muscles from the hypothyroid group, which contain a higher percentage of FOG fibres and consequently a lower percentage of FG fibres. However as with the soleus muscle groups, there is no strong correlation (within each group or when the two groups are combined) of myofibrillar ATPase activity with the percentage of FOG fibres.

Although no strong correlation of myofibrillar ATPase activity with the percentage of FOG fibres exists within either of the four groups, this could be a consequence of the low number of observations, but the more likely explanation is that a simple correlation probably does not exist between the biochemically determined ATPase reaction and the histochemically determined ATPase reaction, as reflected by an increased number of fast fibres.

It is possible that thyroid hormone levels may induce changes in the contractile proteins which do not show up histochemically. For, it is known that the histochemical ATPase reaction is determined by the predominant MHC (Staron & Pette, 1987a, b) and thus light chains which are known to change with dysthyreosis (e.g., Fitzsimons et al.,

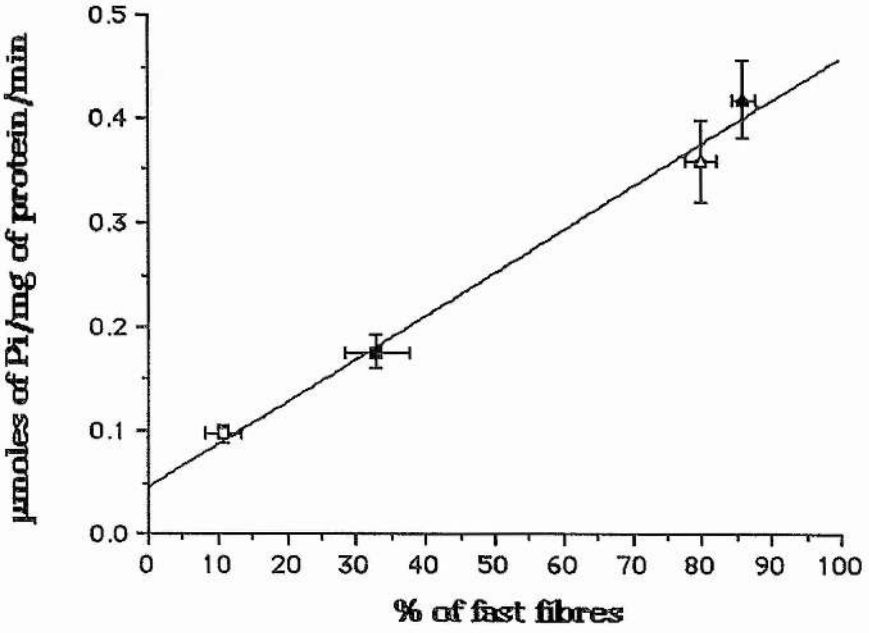
1990) have no effect on the histochemically determined ATPase reaction. Whereas, it is possible that these changes in light chains modulate the biochemically determined ATPase reaction. In this respect, it has been shown that a 50% reduction in the MLC_{2f} content of a muscle reduces the actomyosin ATPase activity by 15-20%. (Moss et al., 1982)

Therefore, giving rise to the possibility that the biochemically determined ATPase reaction can be modulated independently of the histochemically determined ATPase reaction. (cf. with increase in V_{max} without a change in the fibre phenotype as determined histochemically)

However, it must be borne in mind that on a general basis an increase in the histochemically determined ATPase reaction, reflected by an increased percentage of fast fibres, leads to a general increase in the biochemically determined ATPase reaction as is seen when the average myofibrillar ATPase activity is plotted against the average percentage of fast fibres i.e., FOG fibres in the soleus and FG fibres in the TFL (Fig 8.3). It shows a very strong correlation of the two parameters with a correlation coefficient of 0.991 and a slope of 4.13×10^{-3}.

Thus, this suggests that, although the correlation of biochemically determined ATPase reaction with the histochemically determined ATPase reaction might not be apparent on an individual basis, this correlation between the two parameters exists at the group level.

Fig. 8.3: Plot of the average Mg^{2+} activated Ca^{2+} regulated myofibrillar ATPase activity vs. average percentage of fast fibres for hyper- (■) and hypothyroid soleus (□) muscles and for hyper- (▲) and hypothyroid TFL (△) muscles. The correlation coefficient, r, is 0.991. Values are mean ± SEM. Each point represents data from between 4 and 6 animals.



CONCLUSIONS

CONCLUSIONS

The main finding of this project has been that significant differences in maximal isometric tension, maximum velocity of shortening and T/pCa relationships of single skinned fibres from mildly hypo- and hyperthyroid rats have been observed. Moreover, these changes have been observed in the same fibre type as identified histochemically. Furthermore, these changes occurred to a similar extent in both the soleus and TFL muscle fibres.

The increased maximal isometric tension and decreased maximum velocity of shortening of hypothyroid fibres in comparison to hyperthyroid fibres resembles the pattern observed when comparing euthyroid soleus fibres with euthyroid TFL fibres. Moreover, the observed right hand shift in the T/pCa relationship of hyperthyroid soleus muscle fibres with respect to hypothyroid soleus muscle fibres resembles the reported (e.g. Danieli-Betto et al., 1990) right hand shift of fast muscle fibres with respect to slow muscle fibres, along with other characteristics such as the steepness of the T/pCa relationship.

Therefore, a tentative hypothesis based on these results is that mild hypo- and hyperthyroidism changes the dynamic equilibrium of the regulatory and contractile proteins from slow to fast in the direction hypo- to hyperthyroid and from fast to slow in the direction hyper- to hypothyroid. More specifically it is suggested that different isoforms of myosin light chains are mainly responsible for the changes in maximal isometric tension and maximum velocity of shortening, since these changes occurred in the same type of fibre as identified histochemically.

Further evidence for this hypothesis comes from experiments undertaken in conditions of acidic pH and increased inorganic phosphate concentration.

In conditions of acidic pH (pH 6.6), the results in comparison to neutral pH for hypo- and hyperthyroid fibres were generally consistent with those reported (e.g. Fabiato & Fabiato, 1978; Cooke et al., 1988) in euthyroid fibres, i.e. a decrease in maximal isometric tension and maximum velocity of shortening, and a shift to the right for the T/pCa relationship.

Similarly, at increased inorganic phosphate concentration (7.5mM P_i) the results in comparison to normal conditions (i.e. 0mM) for hypo- and hyperthyroid fibres were generally consistent with those reported (e.g. Brandt et al., 1982; Cooke et al., 1988) in euthyroid fibres, i.e. a decrease in maximal isometric tension, a shift to the right for the T/pCa relationship and an unchanged maximum velocity of shortening.

Thus, these results in conditions mimicking fatigue are consistent with a change in the dynamic equilibrium of the regulatory and contractile proteins.

An interesting result was the effect of supra maximal calcium concentrations to reduce maximal isometric tension of both soleus and TFL muscle fibres in the presence of either inorganic phosphate concentration (7.5mM) or reduced pH (pH 6.6). This effect of calcium was probably unmasked due to the effect of dysthyreosis and it may use a mechanism (most probably by decreasing the number of active cross bridges) in the fibre to show that further activity will lead to fibre damage. A decrease due to supra maximal calcium concentrations at acidic pH – to a lesser extent – has been reported in cardiac cells (Fabiato & Fabiato, 1976; Godt & Nosek, 1989) but not in muscle fibres,

On the whole muscle level, myofibrillar ATPase activity was found to be higher in hyper- as opposed to hypothyroid muscles irrespective of muscle type. It was also found that at the group level a higher myofibrillar ATPase activity in hyperthyroid soleus muscles correlated

with a greater percentage of FOG fibres, whilst a higher myofibrillar ATPase activity in hyperthyroid TFL muscles correlated with a greater percentage of FG fibres in comparison with the hypothyroid counterparts.

Moreover, the results of myofibrillar ATPase activity correlate well with the maximum velocity of shortening values obtained at the single fibre level. This suggests that the relationship between maximum velocity of shortening and myofibrillar ATPase activity holds in animals which are hypo- or hyperthyroid.

As an hypothesis, it is proposed that within the three broad categories of fibre types as identified histochemically a continuum exists (due to the many possible combinations of the different isoforms of the proteins of the contractile and the regulatory apparatus) and dysthyreosis changes the dynamic equilibrium of this continuum which is reversible since it is known that the euthyroid state can be restored easily from both states (Ramsay, 1974) and due to the plasticity of muscle (Fig 9.1.).

Fig. 9.1: A possible mechanism of change induced by hyper- or hypothyroid treatment.

No matter what the fibre type e.g., whether it is between the SO or FOG type, it will shift to the left in the hypothyroid state and to the right in the hyperthyroid state, thus changing the contractile function of the fibre accordingly. Obviously, if the fibre is at one extreme then further change in that same direction is limited, by the same token change in the other direction is much greater. As to where a fibre lies along the continuum depends on its composition with regards to contractile and regulatory proteins.

One possible mechanism to explain the change seen with dysthyreosis is that the genetic basis for the control of the different proteins involved in deciding where along this continuum a fibre will lie is shared. It is logical that a selective advantage might be gained from a co-ordinated control of regulatory and contractile proteins. Evidence for co-ordinated expression of myosin light chains and troponin subunits in avian skeletal muscle has been reported (Mikawa et al., 1981).

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